DAXX AND RASSF1 DEFINE A NOVEL MITOTIC STRESS CHECKPOINT THAT IS CRITICAL FOR CELLULAR TAXOL RESPONSE

By

CORY R. LINDSAY

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2008

To my parents Archie and Virginia Lindsay

ACKNOWLEDGMENTS

I thank my parents Archie and Virginia for always providing the love, support and encouragement I have needed to pursue my dreams. I thank Mr. Ed Brogie, Dr. Ed Rosa-Molinar and Dr. Russ Rassmussen who, knowingly or not, motivated, inspired and challenged me. I thank Dr. Alexander Ishov for his excellent mentorship, guidance and knowledge.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DAXX AND RASSF1 DEFINE A NOVEL MITOTIC STRESS CHECKPOINT THAT IS CRITICAL FOR CELLULAR TAXOL RESPONSE

By

Cory R. Lindsay

May 2008

Chair: Alexander Ishov
Major: Medical Sciences-Molecular Cell Biology

Taxanes, a family of compounds which includes taxol, are powerful chemotherapy agents used for breast cancer treatment. Large numbers of patients, however, are resistant to these drugs for unknown reasons. Taxol binds and hyper-stabilizes microtubules, but mutations or alterations in tubulin occur very rarely in cancers and cannot itself explain the majority of Taxol-resistance observed in patients. Currently, it is thought that defects in mitotic proteins may affect Taxol sensitivity in cells. Here, Daxx and Rassf1 are identified as novel regulators of cellular Taxol response. Daxx is a ubiquitously expressed and highly conserved nuclear protein with enigmatic roles in transcription and apoptosis. Increased prometaphase index in Daxx deficient embryos and aneuploidy of Daxx knockout cells was observed which suggested a potential function of Daxx in mitosis and cell division. During interphase, Daxx remains a strictly nuclear associated protein localized to PML bodies or heterochromatin. Upon nuclear envelope breakdown, Daxx was found to co-localize and interact with Rassf1 at mitotic spindles. Rassf1 is a cytoplasmic, microtubule-associated protein that is important for normal mitotic progression and cell division. Daxx was also shown to be important for the proper timing and progression of early mitosis. Together, Daxx and Rassf1 define a novel mitotic stress checkpoint that enables cells to

efficiently exit mitosis (and eventually die) when encountered with specific stress stimuli during mitosis, including Taxol. In the absence of Daxx or Rassf1, cells treated with Taxol remain arrested in mitosis due to a sustained mitotic spindle checkpoint. Upon Taxol decay or removal, these cells can resume mitosis and complete cell division—thus being Taxol resistant. Inhibition of the spindle checkpoint using Aurora Kinase inhibitors efficiently abolished Taxol resistance in Daxx and Rassf1 depleted cells. Deregulation of most known mitotic proteins leads to enhanced Taxol response. Absence or depletion of Daxx and Rassf1, in contrast, results in increased drug resistance. In the future, Daxx and Rassf1 may be useful predictive markers for the proper selection of patients for taxane chemotherapy.

CHAPTER 1
INTRODUCTION

Breast Cancer and Chemotherapy Resistance

Breast cancer is the most frequently diagnosed malignancy for women in the United States. In 2005, an estimated 211,000 new cases of invasive breast cancer were expected to occur (Cancer Facts and Figures 2005, American Cancer Society). The mortality rate from breast cancer declined approximately 2.3% per year from 1990 to 2001, mostly due to earlier detection and improved therapies. Nonetheless, an estimated 40,000 women would die of breast cancer in the United States in 2005 (Cancer Facts and Figures 2005, American Cancer Society). Chemotherapy is a very popular treatment option for many breast cancer patients. There are a number of agents used in adjuvant therapy with established cytotoxic activity, with the taxanes considered some of the most active (O'Shaughnessy, 2005).

Taxanes and their Activity

Taxanes, the group of cytotoxic drugs that includes paclitaxel (taxol) and docetaxol (taxotere), are among the most powerful anticancer agents for breast cancer chemotherapy. Increasing numbers of patients have been treated with these drugs along, or in combination, with other chemotherapeutic agents. The successful entry of paclitaxel into clinical trials in 1986 boosted an interest in understanding the mechanism of taxane-induced cell death and in studying pathways and proteins targeted by this treatment, including tubulin as an immediate target (Schiff et al., 1979) and downstream targets of taxanes, including mitotic proteins (Wood et al., 2001).

Although taxanes are successful in selective killing of tumor cells in clinical settings, current understanding of the molecular basis of this therapy is controversial and incomplete. For

a long time, apoptosis was considered the main mechanism of cell death in response to taxane treatment. Currently, a more distinct model of therapy response is considered, wherein different modes of tumor cell death are likely determined by drug concentration and genetic background of the cells within a tumor (Morse et al., 2005). At pharmacological concentrations, taxanes reversibly bind to tubulin heterodimers that form microtubules—this accelerates polymerization and inhibits depolymerization of tubulin, thus disrupting microtubule dynamics. This event, in turn, activates the spindle checkpoint, which invokes mitotic arrest that in normal, untreated conditions ensures proper chromosomal attachment and alignment and ensures faithful chromosomal segregation preventing aneuploidy (Chan and Yen, 2003; Cleveland et al., 2003). This mitotic arrest does not persist indefinitely. After some period of time, cells usually undergo an aberrant exit from mitosis, characterized by the lack of metaphase, anaphase and cytokinesis. The nuclear envelope is reformed around individual or groups of chromosomes producing large nonviable cells with multiple micronuclei, which are easily distinguishable morphologically from apoptotic cells. Apoptotic cells will have small, highly condensed chromatin with fragmented nuclei and a diminished cytoplasm, whereas micronucleated cells are much larger with uncondensed chromatin in a pattern reminiscent of normal nuclei. This type of cell death which results in micronucleated cells is known as mitotic catastrophe and is activated during mitosis as a result of deranged spindle formation coupled with blocks of different checkpoint mechanisms that activate aberrant chromosome segregation and nuclear fragmentation (Kroemer and Martin, 2005). Taxane-sensitive human breast cancer cells are blocked in mitosis only transiently, followed by nuclear fragmentation and mitotic catastrophe, while resistant breast cancer cells have more prolonged mitotic block and continue proliferation after drug decay and microtubule dynamics restoration, thus surviving chemotherapy (Figure 1-1).

Taxanes and Mitotic Checkpoints

Several mitotic checkpoint proteins, including MPS1, Survivin, Chfr, and members of Mad and Bub protein families (Mad1, Mad2, Mad3 (or BubR1) Bub1, Bub3), sense improper tension between kinetochores and microtubules of the mitotic spindle and transmit a signal to inhibit mitotic progression. Inactivation of most of these checkpoint proteins increases sensitivity to taxane treatment (Carvalho et al., 2003; Lee et al., 2004; Lens et al., 2003). The factors that determine prolongation of mitotic block and, thus, resistance to treatment by taxanes, remain incompletely characterized. To date, only few examples are known when inactivation of mitotic checkpoint proteins leads to reduced sensitivity to taxanes. Inactivation of Chfr, a mitotic-associated E3 ubiquitin ligase (Bothos et al., 2003; Chaturvedi et al., 2002; Kang et al., 2002) which degrades the mitotic kinase Aurora A (Yu et al., 2005) leads to decreased sensitivity to the microtubule depolymerizing drug nocadozole (Scolnick and Halazonetis, 2000). Down-regulation of breast cancer susceptibility gene 1 (BRCA1) by siRNA leads to increased taxane resistance in breast cancer cell line MCF-7 (Chabalier et al., 2006). Another report describes nocodazole-induced delay in mitotic exit upon depletion of $p31^{comet}$ in HeLa cells. $p31^{comet}$ acts in mitosis by counteracting spindle checkpoint function of Mad2 (Xia et al., 2004). Thus, recent efforts have started to link sensitivity of tumor cells to taxane treatment with genetic defects in the cell cycle checkpoints in association with cancer chemotherapy. It has been suggested that inactivation of mitotic checkpoint proteins can contribute to the selective response of taxane treatment *in vivo* (Wassmann and Benezra, 2001). However, mutations in known checkpoint proteins occur rather rarely (Cahill et al., 1998; Haruki et al., 2001); thus broader studies are necessary to search for novel molecular targets of taxane therapy.

Cancer Cell Line Response to Taxane Treatment

Breast cancers are often resistant to the therapeutic induction of apoptosis which is likely due to inactivation of apoptotic pathways (Brown and Wouters, 1999). Therefore, therapies that promote other types of death, such as mitotic catastrophe, may be preferential for use in treating breast cancer. Apoptosis was commonly regarded as the major mechanism of cell death in response to taxanes (Wang et al., 1999); lately, both paclitaxel and docetaxel are observed to induce dose- and cell line–specific apoptotic or mitotic cell death (Roninson et al., 2001). In breast cancer cells and non–small cell lung carcinoma cell lines, each of which originate from supposedly primary targets of taxane therapy in vivo—paclitaxel has a concentration-dependent, biphasic response. At low, pharmacologically relevant concentrations, mitotic catastrophe is observed, whereas at high concentrations terminal cell cycle arrest followed by necrosis are documented (Yeung et al., 1999). Taxane-sensitive human breast cancer cells are blocked in mitosis only transiently, followed by nuclear fragmentation and mitotic catastrophe, while resistant breast cancer cells have more prolonged mitotic block and continue proliferation after drug decay and restoration of microtubule dynamics, thus surviving chemotherapy. The factors that determine prolongation of mitotic block and, thus, resistance to treatment by taxanes, still need to be characterized (Wood et al., 2001).

Predictive Markers for Taxane Treatment

Significant numbers of patients are resistant to taxanes or become resistant to this therapy during treatment. The response rate of docetaxel is ~50% even in the first-line chemotherapy and it decreases to 20-30% in the second- or third-line therapy (Bonneterre et al., 1999; Crown et al., 2004; Ravdin et al., 2003). Together with side effects, which includes peripheral neurotoxicity (Rowinsky et al., 1993), it is of vital importance to select responsive patients using prognosis and

predictive markers. Overcoming resistance or incomplete response to these agents would represent a major advantage in the clinical treatment of breast cancer (Aapro, 2001; Henderson et al., 2003).

A number of studies have been carried out to determine a genomic profile that could be predictive for taxane treatment. In 2003, the group of Dr. Chang published a study of gene expression profiles of 24 patients before and after four cycles of docetaxel treatment in correlation with differential response to chemotherapy (Chang et al., 2003). They observed a differential pattern of 92 genes correlating with docetaxel response allowing the predictive classification of tumor sensitivity. Later this group observed, in the same cohort of patients, chemotherapy-driven positive selection of resistant cells populated by genes involved in G2/M arrest (Chang et al., 2005b). Dr. Kato's group performed a similar study analyzing the expression profile of 44 breast tumor specimens before treatment with docetaxel in combination with clinical response to therapy, and developed a diagnostic algorithm to differentiate between responders and non-responders. They also described elevated expression of redox controlling genes in non-responding patients (Iwao-Koizumi et al., 2005). The same group described down-regulation of aromatase in docetaxel response patients thus connecting this type of chemotherapy with suppression of intra-tumoral estradiol synthesis (Miyoshi et al., 2004). A study comparing gene profiles before and after chemotherapy by either doxorubicin/cyclophosphamide or doxorubicin/docetaxel treatment could not detect a significant profile for the prediction analysis of this combination of chemotherapies probably due to a relatively small group of patients involved (Hannemann et al., 2005). Despite extensive studies trying to identify predictive markers for taxane treatment, the clinical application of results have, so far, been limited, partly

due to uncertainties about the reproducibility of methods between several groups (Mauriac et al., 2005).

Daxx: The Story of an Enigmatic Protein

Daxx and Apoptosis

Daxx is a 120 kDa ubiquitously expressed protein with a high degree of similarity between mice and humans (72% identical by amino acid sequence) (Kiriakidou et al., 1997). Daxx was initially identified through yeast two-hybrid screens as a Fas-interacting protein (Yang et al., 1997). In this initial study, mouse Daxx was found to potentiate apoptosis through a novel pathway involving activation of the jun N-terminal kinase (JNK) and not through an interaction with the Fas-associated death domain (FADD). A follow-up investigation published a year later, showed mDaxx could activate the JNK kinase kinase (ASK1) by binding ASK1 and subsequently relieving an inhibitory intramolecular interaction between the N & C-termini of the protein (Chang et al., 1998).

Through these first two studies, ideas towards the role of Daxx were predominately shifted towards activation of apoptosis and promoting cell death. Perlman et al. (2001) were able to add further weight to the notion of Daxx as a pro-apoptotic molecule by showing it could both physically and biochemically interact with transforming growth factor- β receptor (TGF-β) and aid its apoptotic response by inducing JNK activation. Correspondingly, when antisense Daxx oligo-nucleotides were transfected into AML-12 cells, subsequent TGF-β treatment did not induce apoptosis (Perlman et al., 2001).

Seemingly contradictory evidence towards the role of Daxx in vivo began accumulating a couple of years after its initial discovery. The Leder group developed a Daxx knockout showing a phenotype of extensive apoptosis and lethality by embryonic day 8.5-9, rather than a mouse

with proliferation abnormalities indicative of a pro-apoptotic gene (Michaelson et al., 1999). These results suggested that Daxx supported a role in an anti-apoptotic function. Indeed, knockdown of Daxx expression by RNA interference revealed increased levels of apoptosis as measured by FACS analysis (Michaelson and Leder, 2003). Conversely, it had been known for years that over-expression of Daxx would lead to induction of apoptosis as well (Torii et al., 1999; Yang et al., 1997). What could be the true function of Daxx, in relation to apoptosis, *in vivo*?

Stronger evidence towards the role Daxx could be playing in apoptosis came from studies focusing on the endogenous localization of Daxx in cells. Ishov and colleagues and other groups afterwards (Croxton et al., 2006; Ishov et al., 1999; Ishov et al., 2004; Zhong et al., 2000), found Daxx to interact with the promyelocytic leukemia (PML) tumor suppressor protein and could be subsequently recruited to sub-nuclear domains called ND10 (PML bodies, PODs, or Kraemer bodies) upon sumoylation of PML. An apparent nuclear localization of Daxx, as would be consistently shown by biochemical fractionation and immunofluoresence experiments, raised concern on how Daxx could be involved in Fas-induced apoptosis if Fas was anchored to the cell membrane. A study published shortly after the discovery of a Daxx/PML interaction and ND10 localization showed that human Daxx, although a potent enhancer of Fas-induced apoptosis when over-expressed, did not associate with human Fas in cells and maintained its nuclear localization (at ND10) even upon stimulation of Fas-induced apoptosis (Torii et al., 1999). Moreover, the localization of Daxx to ND10 seemed to be critical for enhancing apoptosis as a Daxx mutant lacking its nuclear localization sequence (and hence its association with PML) was not as effective at promoting cell death (Torii et al., 1999). Zhong and colleagues also supported this claim by showing a larger induction of apoptosis (as measured by TUNEL assay) by Daxx in

$PML^{+/+}$ compared to $PML^{-/-}$ cells (Zhong et al., 2000). Thus, the localization of Daxx to ND10 and not to the cytoplasm was critical for Daxx-enhanced apoptosis.

Daxx and Transcription

The identification of PML interacting with and sequestering Daxx into nuclear domains would become as important a discovery as any study demonstrating the functionality of Daxx. Ishov and colleagues showed that in situations where PML was absent, Daxx would be relocated to condensed heterochromatin where it could potentially be involved in some biochemical function (Ishov et al., 2004). Subsequent studies provided some evidence of what Daxx could be doing at these sites by showing it could interact not only with core histones, but histone deacetylase II (HDACII), Dek (Hollenbach et al., 2002) and the SWI/SNF chromatin remodeling protein ATRX (Xue et al., 2003). These interactions, among others, brought forth the idea that Daxx could be acting as a regulator of transcription, not only on the level of repression but activation as well. Among a few of the many genes Daxx has been implicated in regulating include p53 target genes (Gostissa et al., 2004; Zhao et al., 2004), Pax transcription factor family members (Emelyanov et al., 2002; Hollenbach et al., 2002; Lehembre et al., 2001) and Smad4 (Chang et al., 2005a)

A more dynamic role of Daxx became appreciated when Ishov and colleagues showed a cell cycle dependent localization of Daxx between ND10 and heterochromatin (Figure 1-2) (Ishov et al., 2004). They found that during G1 and G2 phase, Daxx could be found in its characteristic location at ND10, while during S phase, Daxx would relocate to condensed heterochromatin. Interestingly during mitosis, ND10 is disassembled, PML de-sumoylated and Daxx no longer associated with the remnants of these nuclear domains (Ishov et al., 2004). What became the fate of Daxx after this set of events was not addressed.

From the cell cycle-dependent localization of Daxx model which Ishov and colleagues proposed, ND10 could be considered a site of inactivation of Daxx function—a potential "storage depot" for Daxx and numerous other proteins until specific times when they are needed and become active again (Ishov et al., 2004). Although at the time this was not a novel concept, a study conducted by (Li et al., 2000) and similarly by (Lin et al., 2003) suggested this notion showing that when Daxx was bound to increasing amounts of PML, the transcriptional repression activity of Daxx—as measured by a luciferase reporter assay—was relieved. A possible mechanism which could regulate the localization of Daxx to ND10 or to heterochromatin was shown by Ecsedy and colleagues when they demonstrated a physical interaction between Daxx and the serine/threonine kinase HIPK1 (Ecsedy et al., 2003). This interaction was capable of displacing Daxx from PML and re-localizing it elsewhere in the nucleus. In addition, the Ecesedy group found that upon phosphorylation of Daxx by HIPK1, Daxx transcriptional repression activity was modified (Ecsedy et al., 2003). The investigators could not, however, definitively show a relocalization of Daxx to heterochromatin but rather an association with HDAC1. Additionally, they showed that phosphorylation of Daxx by HIPK1 diminished the transcriptionally repressive activity of Daxx rather than enhanced it. Other studies, which focused on the condition-dependent localization of Daxx (and other nuclear body associated proteins) were found to be dependent on the sumoylation status of PML as well as cellular stresses such as heat shock and heavy metal exposure (Nefkens et al., 2003). The small ubiquitin-like modifier (SUMO), moreover, is a post-translational modification added to proteins which affects their function and localization. SUMO bears a 20% identity to ubiquitin and is covalently linked to a wide range of proteins whose functions are commonly implicated in chromatin organization, transcription and genomic stability (Hay, 2005). Although other

conditions may be found to regulate Daxx localization, it remains intuitive that cellular factor(s) and protein modifications play an important role in the regulation of Daxx inside of the nucleus.

To date, Daxx is a protein that has been identified numerous times through yeast two-hybrid screens with various other proteins both as "prey" or "bait." A list of proteins which have been found or used in this way is steadily growing. In some instances, this may be an indication that Daxx could be a false positive of the experimental system. Yet the truly diverse function of Daxx-mediated protein interactions has made elucidating the role of Daxx and its biological significance difficult. The first Daxx-deficient mouse model developed by Michaelson and colleagues still showed the transcription of a mutant form of Daxx, specifically the C-terminal 479 amino acids of the protein (Michaelson et al., 1999). At least theoretically, this C terminal fragment could be responsible for the observed levels of apoptosis and other phenomenon associated with Daxx-deficiency. A more comprehensive Daxx knockout was generated by the Ishov Lab, however, which showed a similar phenotype (Ishov et al., 2004). By embryonic day 8, $Daxx^{-/-}$ mice were developmentally retarded and by day 11.5-12.5, embryos distenegrated completely (Ishov et al., 2004). As we continue to learn more of Daxx biology, we will continually add more to what we already know as a truly unique protein with diverse cellular functions.

Cellular Localization of Daxx

The sub-cellular localization of Daxx has been a controversy since it was discovered as a factor involved in Fas-induced apoptosis. Daxx was identified via yeast-two hybrid screening using Fas as "bait." While Daxx was not completely characterized from this screening, these findings thrust forward the ideology that Daxx would be found as a cytoplasmic-oriented protein near the cell membrane. A subsequent paradox would ensue when Daxx was discovered as a

predominately nuclear protein. Beginning with the study by Pluta and colleagues, which characterized the interaction between centrosome component CENP-C and the human form of Daxx from HeLa cells, large-scale biochemical separation into cytosolic, nuclear and mitotic chromosome fractions would show that Daxx was a protein associated largely with nuclear isolated fractions. Immunofluorescence of endogenous Daxx was described as a "punctuate staining pattern" in interphase nuclei (Pluta et al., 1998). This characteristic Daxx-staining pattern emphasizes its association with PML bodies. Subsequent studies would attempt to validate Daxx interaction with apoptosis signal-regulating kinase 1 (ASK1) and show co-localization and interaction of the two proteins in the cytoplasm, but the bulk of these experiments were based on transient over-expression and this may not necessarily represent the behavior of endogenous proteins (Ko et al., 2001). One report by Lalioti et al. showed very detailed cellular fractionation of NIH-3T3 fibroblasts into nuclear, cytosolic, low-density microsome, high-density microsome and plasma membrane fractions, with the majority of Daxx accumulating in the nuclear fraction and a small percentage appearing in low-density microsomes (Lalioti et al., 2002). Using human Daxx antibody directed against endogenous protein, the Lalioti group observed along with nuclear staining, a very faint speckle-like cytoplasmic Daxx pattern in human fibroblasts which presumed there may be two intracellular pools of Daxx that exist in cells. Strong endogenous interaction between Daxx and other nuclear-associated proteins including chromatin remodeling proteins ATRX and HDACII, nuclear sub-domain constituent PML, nuclear protein kinase HIPK1 among others, suggests Daxx is predominately a nuclear protein. In the absence of PML, the major Daxx housing domain in interphase, Daxx adopts a primarily chromatin-based localization in the nucleus

(Ishov et al., 2004). Thus, if Daxx protein resides in the cytoplasm at any period of time, it would most likely occur as a result of specific relocation as part of signaling pathways.

Several reports describe detailed mechanisms of Daxx re-localization under various stress conditions (Jung et al., 2008; Jung et al., 2007; Junn et al., 2005; Karunakaran et al., 2007; Song and Lee, 2003). In many cases, this change in distribution of Daxx was shown to be critical for cell survival under stress. During glucose deprivation, Daxx is re-located from the nucleus to the cytoplasm (Song and Lee, 2003, 2004). Mutation of Trpytophan 621 and Serine 667 of human Daxx, moreover, was sufficient to block nuclear export in these stress conditions, which relied on stable adenoviral expression of Daxx in adenocarcinoma DU-145 cells. Chemical hypoxia-induced Daxx relocalization to the cytoplasm was eloquently shown by (Jung et al., 2008) using detailed confocal imaging analysis of endogenous Daxx in Chinese hamster ovary cell line PS120. Oxidative stress was also reported to influence the localization of Daxx to the cytoplasm in DU-145 cells, while over-expression of catalase inhibited nuclear export of Daxx and its glucose deprivation-induced cytotoxicity (Song and Lee, 2003). A contradictory report by (Khelifi et al., 2005) however, showed via biochemical separation that Daxx remains in the nucleus after exposure to hydrogen peroxide or UV treatment. In the majority of studies, however, the primary means of determining stress induced Daxx localization was accomplished by immunofluorescence staining of transiently over-expressed protein. In some cases, these localization patterns may be a result of artifacts—created either by conditions of treatment or methods of fixing and staining of cells. Alternatively, many observed properties of Daxx in response to stress stimuli may be cell-line specific. Can stress-induced Daxx re-localization be explained by the shuttling of other nuclear proteins into the cytoplasm? To date, few studies have effectively incorporated these controls into their investigations. If a general re-distribution

of nuclear proteins is observed in these cases, it is possible that these phenomena are less attributable to Daxx function and more explainable as a general cellular stress response. Reports such as (Nefkens et al., 2003) for example, may describe more functional stress-induced Daxx redistribution in that the activity of several nuclear- and ND10-associated proteins were documented in parallel after response to specific stress stimuli. Specifically, Daxx and Sp100 but not PML would disperse from ND10 into the nucleoplasm due to rapid desumoylation of PML during heat shock, while heavy metal exposure would release Daxx and PML with Sp100 retained at ND10 (Nefkens et al., 2003). While there is some tantalizing evidence to suggest existence and function of Daxx in the cytoplasm, more extensive studies of endogenous protein trafficking are required.

Ras-Association Domain Family-1 (Rassf1) and Cancer

The Rassf1 gene locus comprises approximately 11,151 base pairs of the human genome and consists of eight exons (Agathanggelou et al., 2005). It is found on the short arm of human chromosome 3 (3p21.3). Differential promoter use and alternative splicing creates seven transcripts Rassf1A-Rassf1G. Of these transcripts, isoforms Rassf1A and Rassf1C are predominately expressed in all tissues while Rassf1B is expressed in the hemopoeitic system only, and Rassf1D or Rassf1E expression is restricted to cardiac or pancreatic cells, respectively. Homologues of Rassf1A exist in rodents, fish, nematodes and fruit flies ranging from 38% to 85% identity (Agathanggelou et al., 2005). A Rassf1C homologue exists in Xenopus with no apparent homologue for Rassf1A.

Preferential Alteration of Rassf1A in Cancer

Allelic loss of the short arm of human chromosome 3 (3p) is one of the most frequently occurring events in lung cancers (90% of small cell lung cancers 50-80% of non-small cell lung cancers) (Dammann et al., 2000). Specifically, the region 3p21.3, where the Rassf1 locus resides, displays regular loss of heterozygosity and homozygous deletions in cancer cells. Promoter methylation and loss of protein expression have been directly correlated specifically to Rassf1A in many different tumor cell lines and this methylation has been confirmed in at least 37 different tumor types (Agathanggelou et al., 2005). Methylation of the Rassf1A promoter does not affect expression of Rassf1C and in numerous instances Rassf1C is used as a control for RNA integrity and loading when studying Rassf1A expression. Therefore, epigenetic inactivation of Rassf1C is much rarer than that of Rassf1A. In one study, Rassf1A promoter methylation occurred with a frequency of 62% in forty-five breast carcinomas that were analyzed and in many instances treatment of cells with the DNA methylation inhibitor 5-aza-2`-deoxycytidine reactivated Rassf1A transcript expression (Dammann et al., 2001). Because of this very strong correlation between loss of Rassf1A expression and tumor-specific cell lines it is highly regarded as a candidate molecular marker for tumor diagnosis. Differential loss of Rassf1C expression, however, has been documented in some tumor cell lines and may be regulated by more significant post-transcriptional mechanisms than Rassf1A (Donninger et al., 2007).

Rassf1A and Cell Cycle Control

Because of the intense focus of Rassf1A inactivation in cancers, the majority of functional studies about Rassf1 have swayed largely to the A isoform of Rassf1 because of its potential tumor suppressor roles. Indeed, a seminal observation that has been repeatedly confirmed for

Rassf1A as a tumor suppressor is the reintroduction of the protein by over-expression reduces colony formation in soft agar assays, suppresses growth and reduces independence of anchorage-free cell growth (Burbee et al., 2001; Dammann et al., 2000). Along with reports of growth regulation was the first observations that Rassf1A could block cell cycle progression. Over-expression of Rassf1A was found not to impact apoptosis, but to block cells strongly in the G1 stage of interphase and inhibit accumulation of cyclin D1 (Shivakumar et al., 2002). Importantly, these findings also showed that Rassf1A transcript variants (identified from breast and lung tumor samples) could not block cells in G1 compared to wild-type Rassf1A (Shivakumar et al., 2002). Supporting the evidence of Rassf1A function in G1-arrest was a study by the Fenton group showing interaction of $p120^{E4F}$ with Rassf1A that was necessary for G1-arrest (Fenton et al., 2004). Furthermore, this function became even more intricate when Song and colleagues described a dynamic cell cycle-dependent regulation of Rassf1A protein levels by Skp2 ubiquitin ligase complex (Song et al., 2007). When Rassf1A levels were degraded by targeted ubiquitination, it was shown that cells were able to sufficiently progress through G1 into S phase. Subsequent studies would also begin to analyze sub-cellular localization of Rassf1A and associate its localization in cells with function.

The Pfeifer group were the first to describe Rassf1A as a protein that co-localizes with microtubules during interphase and associates with the spindle apparatus during mitosis (Liu et al., 2003). By using $Rassf1^{-/-}$ cells and over-expression studies, it was shown that Rassf1A provided stability to microtubules and the region of tubulin interaction was mapped to a specific 169 amino acid stretch of the carboxy-terminus of Rassf1A (Liu et al., 2003). The Vos and Dallol groups would confirm Rassf1A-association with microtubules and microtubule-associated proteins and that these interactions were important for microtubule stability, dynamics and

preventing genome instability (Dallol et al., 2004; Vos et al., 2004). Liu and colleagues also described that overexpression of Rassf1A induced aberrant mitotic arrest at metaphase in a similar manner to the microtubule stabilizing drug taxol and how it affects cells (Liu et al., 2003). This became the first study to link Rassf1A to possible mitotic functions in cells.

Additional insight into the role of Rassf1A function in mitosis and cell cycle progression came in the seminal study by (Song et al., 2004). Rassf1A was shown to influence the stability of both Cyclin A and Cyclin B as a result of direct interaction with Cdc20 and negative regulation of the anaphase promoting complex (APC). Without Rassf1A, cells were proven to progress through early mitosis (specifically pro-metaphase) faster as a result of premature activation of APC. Absence of Rassf1A by siRNA depletion also caused centrosome abnormalities and multipolar spindles. Details of interaction of Rassf1A with Cdc20 would later become a subject of controversy as Liu and colleagues showed that Rassf1A was not capable of interacting with Cdc20 *in vitro* and immunoprecipitation of Cdc20 with Rassf1A could not be detected in synchronous or asynchronous cells (Liu et al., 2007). In the future, additional studies into the precise role of Rassf1A regulation of mitosis will be required.

Cellular Localization of Rassf1A

Not until the Pfeifer group described co-localization of Rassf1A with microtubules did the scientific community know about the distribution of the protein in cells (Liu et al., 2003). While this study showed ample evidence of the microtubule-associated network that over-expressed Rassf1A will form in cells, the Vos group were the first to describe the endogenous interaction of Rassf1A with polymerized tubulin (Vos et al., 2004). Depolymerized tubulin was unable to interact with Rassf1A. The same group performed a yeast two-hybrid screen using Rassf1A as "bait" and identified and confirmed several known microtubule-associated proteins as interacting

partners of Rassf1A, including microtubule associated protein 1A (MAP1A), MAP1B and C19ORF5 (Dallol et al., 2004). Several reports have shown because of the direct interaction between Rassf1A, tubulin and tubulin-related proteins, it served to stabilize microtubules under stress conditions (i.e. nocodazole treatment) (Liu et al., 2003; Rong et al., 2004). During mitosis, over-expressed Rassf1A was observed to co-localize with centrosomes and microtubules during prophase, the spindle apparatus (spindle poles and spindle fibers) during prometaphase, metaphase and anaphase and microtubules as they reformed in divided daughter cells (Liu et al., 2003; Song et al., 2004). The minimal interaction domain that is responsible for Rassf1A binding to microtubules and for association with the spindle apparatus was mapped to amino acids 120-288 of Rassf1A (Liu et al., 2003).

Functions of Rassf1C

While extensive studies on the epigenetic regulation of Rassf1A have been performed coupled with essential studies into the function of Rassf1A and its importance in tumor progression, very little is known about Rassf1C biology. Armesilla et al. (2004) used a yeast two-hybrid screen to find novel interactors of plasma membrane Ca^{2+} pump 4b (PMCA4b) which identified Rassf1C. The interaction between Rassf1C and PMCA4b was narrowed down to a region that is common to both Rassf1C and Rassf1A, presumably showing that this interaction is shared between both isoforms although this data was not shown. Potential tumor suppressor functions of Rassf1C were first described by Li and colleagues demonstrating that Rassf1C could substantially reduce anchorage independent growth of tumor cells and elicit cell cycle arrest similar to Rassf1A (Li et al., 2004). Amaar et al. performed a similar investigation showing that upon depletion of Rassf1C protein in H1299 cells that lack Rassf1A expression, it caused a significant decrease in cell proliferation (Amaar et al., 2006). Upon over-expression of

Rassf1C, however, cell proliferation was actually increased compared to cells over-expressing Rassf1A. This became some of the first tantalizing evidence to suggest that Rassf1A and Rassf1C could have different effector targets. The microtubule binding and stabilizing functions of Rassf1C were also characterized and shown to have identical properties to Rassf1A-mediated microtubule stability, although these findings were largely an oversight (Rong et al., 2004)

Interestingly, a study purporting the interaction of Rassf1C and Daxx in the nucleus was described by (Kitagawa et al., 2006). This study suggested that upon degradation of Daxx, Rassf1C would be released from the nucleus where it can activate the SAPK/JNK pathway to trigger apoptosis during stress conditions. If proven correct, this evidence and others like it (Amaar et al., 2006) suggest that although Rassf1A and Rassf1C have several properties in common they may also have many divergent roles in the cell.

Cellular Localization of Rassf1C

The localization of Rassf1C in cells has not been a subject of critical attention. Several reports suggested microtubule-associated localization of over-expressed Rassf1C was similar to Rassf1A localization although these similarities were not emphasized (Liu et al., 2002; Liu et al., 2003; Vos et al., 2004). Some reports have suggested over-expressed Rassf1C adopts a nuclear localization, perhaps in an effort to separate the tumor suppressor-based roles of Rassf1A from its lesser isoform (Song et al., 2004). Moreover, the description of both over-expressed and endogenous Rassf1C as a component of PML bodies was described by Kitagawa, et al. (2006). In this study, Rassf1C was shown to be exported from the nucleus when Daxx was degraded by ubiquitination or siRNA depletion, implying a Daxx-dependent nuclear localization of Rassf1C (Kitagawa et al., 2006). More extensive studies into the partitioning of Rassf1C inside of the cell are required.

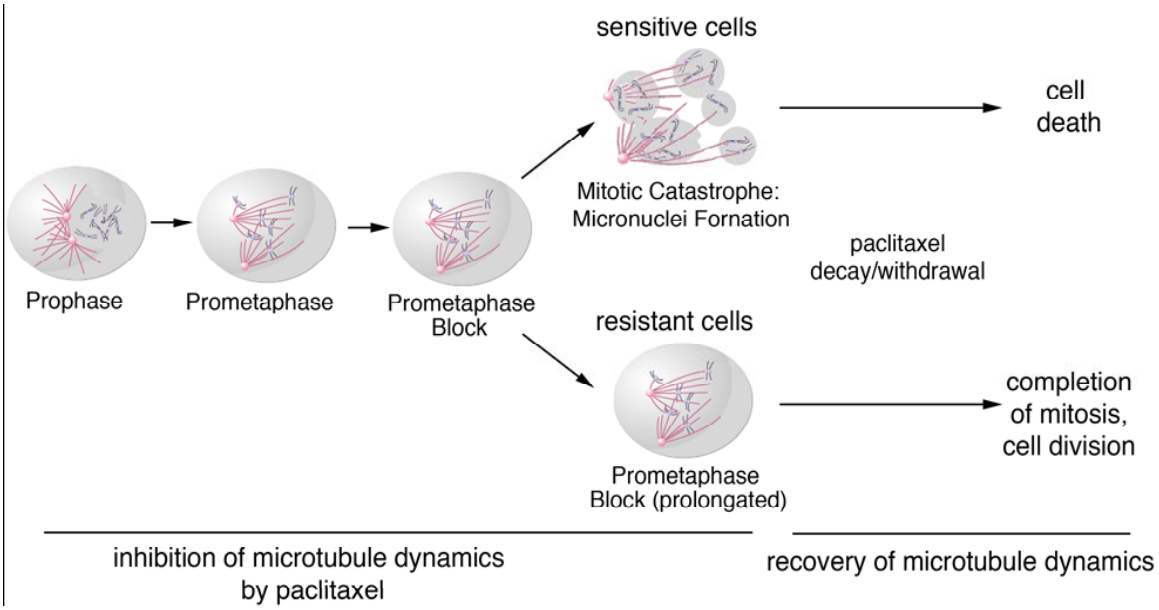


Figure 1-1. Dynamics of Paclitaxel Action in Cells. At pharmacological concentrations, paclitaxel reversibly inhibits microtubule dynamics blocking cells in pro-metaphase. Cells that are sensitive to paclitaxel activate mitotic block only transiently, followed by micronucleation (mitotic catastrophe) and cell death, while resistant cells have a more prolonged pro-metaphase block and continue proliferation after drug decay/ withdrawal and microtubule dynamics restoration—thus surviving chemotherapy.

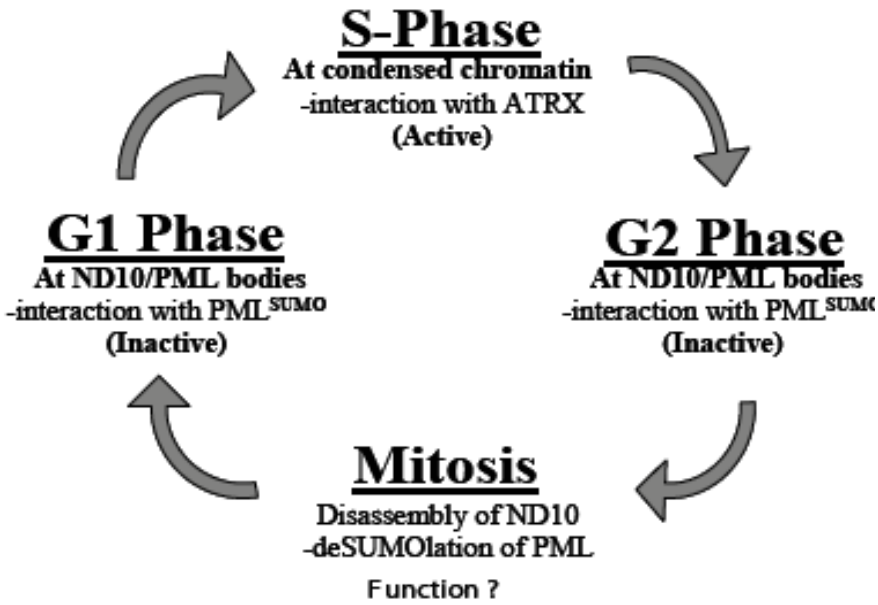


Figure 1-2. Localization of Daxx Throughout the Cell Cycle. During G1 and G2 phase, Daxx is localized to PML bodies where it is presumably inactive. During S phase, Daxx relocates from PML to condensed chromatin where it interacts with ATRX. The fate and function of Daxx during mitosis (M phase) is not documented.

CHAPTER 2
MATERIALS AND METHODS

Antibodies

Antibodies to Daxx (M112), BubR1 (8G1), Bub1 (14H5), Cdc20 (H-175), Cdc27 (AF3.1), Cyclin B (GNS1) and Mad2 (17D10) were from Santa Cruz Biotechnology; GST monoclonal antibody was from Sigma; 6X His monoclonal antibody was from Invitrogen; Rassf1C mouse polyclonal antibody was from UT Southwestern; Rassf1A monoclonal antibody was from Abcam; Rassf1A/C polyclonal antibody was a gift from Dae Sik Lim, Korea Advanced Institute of Technology; PML rabbit polyclonal antibody was from Gerd Maul, Wistar Institute; Phospho Histone H3 (Ser10) rabbit polyclonal antibody was from Upstate; α-Tubulin monoclonal antibody was from Sigma; Daxx monoclonal antibody 514 and rabbit polyclonal antibody 2133/2134 were developed as described in (Ishov et al., 2004).

β-Gal Reporter Assay

Starter cultures of yeast were grown overnight in CM selective media and OD_{600} readings were measured the next day. Cells were resuspended in breaking buffer (100 mM Tris-HCl pH 8.0, 1 mM DTT, 10% glycerol, 40 mM PMSF) and glass beads were added and suspensions were vortexed for approximately 5 min at 4°C. After pelleting cell debris, a 1:10 mixture of supernatant/Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) was made and incubated for 5 min at 28°C. 200 µL ONPG (Sigma) was added to the mixtures and the time taken for reactions to turn yellow was recorded. Afterwards, 400 µL of Na_2CO_3 was added to stop reactions. β–Gal activity was measured at OD_{420} by making 1:10 mixtures of ONPG reaction/water and recording spectrophotometer readings. Protein concentration was determined by OD_{595} spectrophotemeter readings afterterwards.

Biochemical Fractionation

HEp2 cells were separated into nuclear and cytosolic fractions using a biochemical fractionation buffer consisting of 250 mM sucrose, 20 mM HEPES-KOH pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA. Cells were grown on 100 mm dishes and washed 2X with PBS and then placed on ice. Ice cold fractionation buffer was immediately added to dishes and cells were incubated at 4˚C with gentle rocking for 15 minutes. Cells were then collected into tubes and dounced five times with a B-type pestle. Afterwards, cell extract was collected into microcentrifuge tubes and centrifuged for 2-3 min at 800 RPM to separate intact nuclei from soluble cytoplasm fraction. Nuclei were washed 2X with fractionation buffer and resuspended in 1 mL fractionation buffer. 5 M NaCl was then added to the buffer to reach a final concentration of 450 mM NaCl. Nuclei were then placed at 4˚C and subjected to gentle inverting for 5-10 minutes. Nuclear extract was then centrifuged at 13,000 RPM for 5 minutes at 4˚C to pellet insoluble nuclear material.

Cell Culture and Transfections

Daxx +/+ MEFs, Daxx -/- MEFs, HEp2, MDA MB 468 and T47D cells were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin/ streptomycin and maintainted in 5% CO_2 at 37˚C. For transient transfection in mammalian cells, all cDNAs were transfected using the Lipofectamine protocol (Invitrogen) according to the manufacturer's instructions.

Cell Cycle Synchronization

HEp2 and MDA MB 468 cells were synchronized at G1/S using a standard double thymidine block protocol. Briefly, asynchronous cells were set up at 20-30% confluency and treated with 2 mM thymidine for 18-20 hrs, washed 2X with PBS and released into normal media without thymidine for 6 hrs. 2 mM thymidine was then added for an additional 18-20 hrs and cells were washed 2X with PBS and released from G1/S block for experiments.

Colony Formation Assay

Treated and non-treated cells were set up for colony formation using a series of cell dilutions ranging from 1:100 to 1:1000 to optimize colony growth. On average, cells were grown for 5-7 days after set up. Colonies were then washed, fixed with methanol and stained with crystal violet (Fisher) in order to visibly count colonies. Each colony formation assay was set up in triplicate in order to obtain representable statistics for each drug treatment and its duration of exposure to cells.

Confocal Microscopy and Subcellular Localization

HEp2 cells were cultured on cover dishes for 24 hrs and then transfected with GFP-Daxx, GFP-Rassf1A, or GFP-Rassf1C. 24-48 hrs later, live cell confocal images were taken using a Leica TCS SP5 microscope and chamber and Leica imaging software.

Drug Treatment

Standard concentration of paclitaxel (Calbiochem) used throughout these studies is 10 nM, unless noted otherwise. Paclitaxel was used to treat both asynchronously and synchronously growing cells. Standard concentration of nocodazole (Calbiochem) was 10 μM and was used to

treat both asynchronously and synchronously growing cells. Standard concentration of Aurora kinase inhibitors ZM447439 (Tocris Bioscience) and Aurora kinase inhibitor III (Calbiochem) was 10 μM. Aurora kinase inhibitors were used to treat only synchronously growing cells.

Embryo Isolation and Culture

Daxx +/+ and Daxx -/- embryos were collected at E9.5 from timed matings of Daxx +/- mice. Presence of a sperm plug was designated as +0.5 days. Approximately nine days later, female mice were euthanized and embryos (if present) were collected from the uterus. E9.5 embryos were cultured on glass coverslips for immunostaining or paraffin-embedded for tissue sectioning. In each case, material from individual embryos was collected for genotyping to confirm presence or absence of Daxx alleles.

FACS Analysis

Cells were trypsonized and re-suspended in DMEM + 10% FBS + 1% penicillin/ streptomycin, centrifuged and washed 2X with PBS and then fixed in 95% ethanol. After fixation, cells were treated with 0.5 μg/mL RNase for 20 minutes and propidium iodide was added to a final concentration of 20 μg/mL afterwards. Cells were then analyzed via flow cytometry for cell cycle distribution.

Fluorescence Time-Lapse Microscopy

Control and Daxx-depleted HEp2 cells stably transfected with GFP-Histone H3 (gift from Duane Compton, Dartmouth University) were cultured on cover dishes for 24 hrs and then grown at 37˚C in a microscope chamber supplying 5% CO_2. Mitotic cells were imaged using a confocal Leica TCS SP5 microscope and Leica imaging software.

Immunofluorescence

Cells were cultured on glass coverslips in 24-well plates (Corning), washed with KM buffer (100 mM MES, 100 mM NaCl, 120 mM $MgCl_2$, 50% glycerol) and fixed with 1% paraformaldehyde or ice cold methanol at room temperature for 20 min. After treatment with 0.4% Triton X-100 in PBS (paraformaldehyde-fixed cells only), the cells were incubated with primary antibody for 1 hr at room temperature and then washed 2X with PBS and secondary antibodies (FITC or Texas Red-conjugated, Vector Labs) were then applied sequentially for 45 min each at room temperature in the dark. Cells were then stained with HOECHST (Vector Labs) and mounted on slides using Fluormount G (Southern Biotech), dried and analyzed using a Leica fluorescent microscope and imaged using Openlab software.

***In vitro* Pull-down Assay**

Daxx and Rassf1 constructs were cloned into pGEX-2T (Invitrogen), pGEX-4T3 (Invitrogen), and pQE-30 (Qiagen) plasmids, respectively. Constructs were then transformed into a Rosetta strain of *E. coli*. Protein expression was induced using 50 mM IPTG (Fisher) (Daxx constructs) and 100 mM IPTG (Rassf1 contructs) at room temperature for 6 hrs (Daxx) or 18˚ C overnight (Rassf1). Cells were then pelleted and lysed using a lysis buffer consisting of 0.1% Triton X-100, 2 mM phenyl-methyl-sulfonyl fluoride (PMSF) (Calbiochem), 1 μg/mL aprotinin (Sigma), 1 μg/mL leupeptin (Sigma), 1 μg/mL pepstatin (Sigma) and 50 μg/mL 2–mercapto-ethanol (Sigma) in TBS. A GST- and 6X-His pulldown kit (Pierce Biotechnology) was then used to determine binding capability as per the manufacturer's instructions. Protein samples were analyzed on 4-20% Tris-HCl, sodium dodecyl sulfate (SDS)/arcrylamide gels (Biorad).

Plasmid Constructs

GFP-hDaxx wt and deletion mutants were cloned into the BamHI site of pEGFP-C1. GST-hDaxx wt and deletion mutants were cloned into the BamHI site of pGEX-2T and pGEX-4T3. pEGFPC2-hRassf1C was a gift from Gerd Pfeifer, Beckman Research Institute. RFP-mRassf1C wt and deletion mutants were cloned into the EcoRI/HindIII site of pDS-RedN1. 6His-mRassf1C was cloned into pQE-30. GFP-hRassf1A was a gift from Dae Sik Lim, Korea Advanced Institute of Technology. RFP-hRassf1A wt and deletion mutants were cloned into the EcoRI/HindIII site of pDS-RedN1. GST-hRassf1A wt and deletion mutants were cloned into the BamHI site of pGEX-2T and pGEX-4T3.

Stable siRNA Infection

MDA MB 468 and HEp2 cells were transduced with recombinant lentivirus supernatants encoding hairpin siRNA hDaxx, hRassf1A and control expression constructs which were collected from ~5 x 10^4 transfected 293T cells used for multiple rounds of infection in the presence of 4 ug/ml polybrene. The lentiviral expression system was provided by Peter M. Chumakov (Lerner Research Institute, Cleveland; (Sablina et al., 2005). This lentiviral system comprises a targeting envelope expression vector pCMV-VSV-G, a generic packaging expression vector pCMV-deltaR8.2 and the expression cassette for custom siRNA pLSL-GFP that contains a minimal histone H4 promoter that drives transcription of a GFP gene allowing fluorescent cell sorting. Candidate siRNAs for Daxx were designed according to the Dharmacon si*DESIGN* algorithm (http://www.dharmacon.com/sidesign/). Anti-Daxx siRNA 1 was targeted against base pairs 1552-1570 of hDaxx (sense CTACAGATCTCCAATGAAA; anti-sense TTTCATTGGAGATC TGTAG); anti-Daxx siRNA 2 was targeted against base pairs 100-118 of

hDaxx (sense GATGAAGCAGCTGCTCAGC; anti sense GCTCAGCAGCTGCTTCATC); control siRNA was directed against base pairs 1262-1284 of SETDB1 (sense TCCTCTTTCTTATCCTCGTATGT, anti-sense ACATACGAGGATAAGAAAGAGGA).

Western Blotting

Cell and protein extracts were ran on pre-made 4-20% Tris-HCl, SDS/Acrylamide gels (Biorad), separated by electrophoresis and transferred onto nitrocellulose and PVDF membranes (Biorad). Membranes were then blocked using 3% milk in 0.1% PBS-Tween for 30 minutes at room temperature. Primary antibodies were added and incubated overnight at 4˚C. The next day, membranes were washed 3X with 0.1% PBS-Tween and secondary antibodies (mouse and rabbit HRP conjugates, Cell Signaling Technologies) were added and membranes were incubated at room temperature for 1 hr. After 3X wash in PBS-Tween, membranes were then exposed with ECL (Amersham) and developed.

Yeast Two-Hybrid Assay

The bait vector pGBDC1-mDaxx wt, -mDaxx C term and -mDaxx ΔC were transformed into yeast strain PJ-69a and PJ-69 alpha along with mouse E11.5 cDNA library plasmid cloned into pGADC1. Briefly, frozen yeast competent cells were vortexed until melted completely and then incubated at room temperature for 45 min in 1 mL solution B (40% polyethylene glycol 1000, 200 mM bicine pH 8.35). After pelleting cells, supernatant was removed and cells were resuspended in 1 mL solution C (150 mM NaCl, 10 mM bicene pH 8.35) and repeated with a second wash in solution C. Cells were then resuspended in 100 μL solution C and plated on selective media (CM media – tryptophan,- leucine and CM media - tryptophan,- leucine,- histidine).

CHAPTER 3
DAXX FUNCTION IN MITOSIS

Introduction

Components of the mitotic spindle checkpoint, as well as factors involved in the controlled regulation of the spindle checkpoint and other mitotic processes, are necessary for proper partitioning of chromosomes into daughter cells. In the absence of such factors, cells can mishandle proper chromosomal alignment and segregation during metaphase and anaphase, resulting in lagging chromosomes and aneuploidy (Ikui et al., 2005). Proper timing and the rate of mitotic progression can also be affected in the absence of one or more mitotic proteins. For this reason, the cell cycle has been compared to a series of molecular timers: "clocks" that control the average duration of each cycle, and "dominoes" that make each step dependent on the proper completion of a prior step (Meraldi et al., 2004). Cyclin B and Securin are two of the primary mitotic substrates that drive entry into and exit from mitosis (Pines, 2006). Using biochemical analysis of the relative stability of these proteins it is possible to measure the duration of mitosis and cell division. Along with the targeted depletion of proteins and time-lapse microscopy, many mitotic factors have been shown to affect timing and rate of mitotic progression—adding leverage to their already established roles as mitotic regulators and adding the possibility of uncovering novel proteins that may also influence cell cycle progression.

Results and Discussion

Originally identified as a pro-apoptotic Fas-interacting protein and later demonstrated to have anti-apoptotic activity (Michaelson, 2000), Daxx is a ubiquitously expressed and highly conserved nuclear protein that is also implicated in transcription regulation. As an almost exclusively nuclear protein during interphase, Daxx interacts with intrinsic kinetochore protein

CENP-C (Pluta et al., 1998); depletion of Ams2, a Daxx-like motif-containing GATA factor in *S. pombe* results in chromosome mis-segregation (Chen et al., 2003). Extensive aneuploidy was also observed in $Daxx^{-/-}$ MEFs, a typical manifestation of chromosomal mis-segregation in cells (Figure 3-1, bottom). This condition was observed in three independent $Daxx^{-/-}$ MEF cell lines, suggesting Daxx may be important for accurate chromosomal separation and/or cell division. Furthermore, upon paraffin-sectioning of E9.5 Daxx-deficient embryos and HOECHST staining of cells, an increased number of Daxx-deficient cells were observed in early mitosis, specifically pro-metaphase (Figure 3-1, top). In contrast, a smaller number of cells were observed in later stages of mitosis (anaphase, telophase and cytokinesis) compared to corresponding wild type Daxx embryos. These findings suggest a potential involvement of Daxx in mitosis progression.

To understand the extent of Daxx influence on the rate and timing of mitosis, the effect of Daxx-depletion on mitosis progression was analyzed on HEp2 cells which were stably transfected with GFP-Histone H3 and a control siRNA (mouse Daxx) or human Daxx siRNA using fluorescence time-lapse microscopy. Cells expressing human Daxx siRNA were efficiently depleted of Daxx-protein levels, compared to control (Figure 3-2). Photo-toxicity associated with fluorescence microscopy limited the time interval of movies to one frame every 2 minutes. Using the precedent of Meraldi and colleagues, key events in mitosis were monitored and timed based on chromosome condensation to nuclear envelope breakdown (prophase to pro-metaphase), nuclear envelope breakdown to central chromosome alignment (pro-metaphase to metaphase) and chromosome segregation (anaphase) (Meraldi et al., 2004). Results of this analysis are formulated in Table 3-1. Extending from the beginning of chromosomal condensation to nuclear envelope breakdown, completion of prophase in Daxx-depleted cells was on average 31.4% faster (7.05 min +/- 2.64 min in Daxx siRNA, compared to 10.2 min +/-

2.3 min in control siRNA) than control cells (Figure 3-3). Overall, condensation of chromosomes in Daxx-depleted cells occurred more rapidly and the onset of pro-metaphase occurred sooner. The transition from nuclear envelope break down to chromosome segregation in anaphase was also 20.5% slower in Daxx-depleted cells (37.6 min +/- 10.36 min for Daxx siRNA, 31.2 min +/- 7.9 min for control siRNA) (Figure 3-3). Hence, absence of Daxx significantly influences the rate of early mitosis progression in HEp2 cells which emphasizes initial observations of increased pro-metaphase index in Daxx knockout embryos.

To confirm that Daxx protein has a direct effect on cell cycle progression, HEp2 cells stably expressing control or Daxx siRNA were synchronized using a double thymidine block and then released to allow cells to progress through mitosis synchronously so mitotic cyclin B levels could be analyzed. Defective mitotic progression was seen in Daxx-depleted cells as cyclin B levels were consistently sustained longer than wild-type (parental) or control siRNA cells (Figure 3-4). Specifically, during the time point of 9-9.5 hr release from thymidine block in wild type and control siRNA cells the majority of Cyclin B levels are destroyed, however, Cyclin B stability is preserved in Daxx siRNA cells past this time point until 10-10.5 hrs after thymidine release. These data implicate Daxx in the regulation of mitotic progression, either directly as a regulatory mitotic protein or indirectly through other mechanisms.

To rule out possible indirect mechanisms of Daxx regulation of mitosis, protein expression of several known mitotic proteins, including Mad2, a key mitotic checkpoint protein; Cdc20 the activator of the anaphase promoting complex (APC)/cyclosome and Cdc27, a major subunit of the APC was analyzed and compared in control- and two independent Daxx-depleted HEp2 cell lines (Fig 3-5). By comparison, no significant changes in protein expression were observed in the presence or absence of Daxx, which supports microarray analysis from $Daxx^{+/+}$ and $Daxx^{-/-}$

embryos which showed no apparent changes in mitotic protein expression (data not shown). Thus, there is little evidence to suggest Daxx can regulate transcription of known mitotic proteins. The expression of Daxx protein throughout different stages of the cell cycle was also analyzed and found to change insignificantly (Figure 3-6). Thus, Daxx is a very stable protein throughout the cell cycle that has direct involvement in the regulation of mitotic progression.

Cell cycle-dependent protein localization of endogenous Daxx was analyzed in $Daxx^{+/+}$ MEFs as they progressed into and through mitosis (Figure 3-7). A striking mitotic spindle-like association of Daxx was consistently observed in $Daxx^{+/+}$ MEFs which was not present in $Daxx^{-/-}$ cells beginning in pro-metaphase, upon nuclear envelope breakdown. Daxx was observed to localize in the nucleus in prophase typically at PML bodies, but by early pro-metaphase, Daxx protein localization was redistributed to spindle structures as they were being formed (Figure 3-7). By late pro-metaphase, the majority of Daxx was distributed to the spindle apparatus, away from PML. Biochemically, it is known that during mitosis, PML bodies are de-sumoylated and the bulk of PML-associated proteins, including Daxx, leave during this time (Dellaire et al., 2006; Ishov et al., 2004). The spindle-like localization of Daxx is maintained through metaphase, but by the later stages of mitosis (anaphase to cytokinesis) this association is absent. Thus, Daxx is a spindle associated protein that is important for the correct timing of early mitosis progression in cells.

Table 3-1. Statistical Analysis of Mitotic Progression in Control- and Daxx-Depleted H3-GFP-HEp2 Cells. Peak time designates the most frequently occurring time of completion for each stage of mitosis.

Stage	RNAi	Average	Peak time	Standard deviation	Min	Max
Prophase						
	Control	10.2 min	10 min	2.3 min	6 min	18 min
	Daxx	7.5 min	6 min	2.64 min	2 min	14 min
Prometaphase-anaphase						
	Control	31.2 min	28 min	7.9 min	22 min	58 min
	Daxx	37.6 min	32 min	10.36 min	22 min	78 min

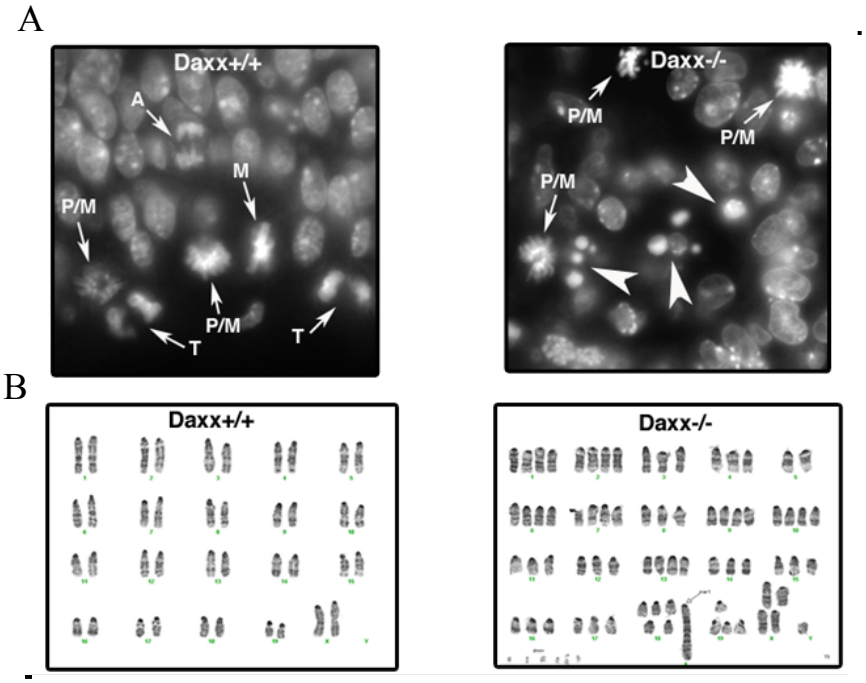


Figure 3-1. Characterization of Daxx$^{-/-}$ Mouse Embryos and Cells. A) HOECHST immunohistochemical staining of Daxx$^{+/+}$ and Daxx$^{-/-}$ E9.5 mouse embryos. Mitotic stages are marked with small arrows. P/M=pro-metaphase, M=metaphase, A=anaphase, T=telophase. Apoptotic cells are marked with large arrowheads. B) Karyotype analysis of Daxx$^{+/+}$ and Daxx$^{-/-}$ MPEFs generated from E9.5 embryos. Daxx$^{-/-}$ MPEFs exhibit aneuploidy.

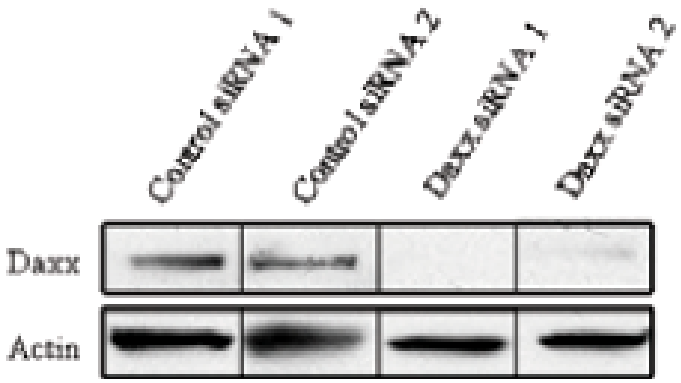


Figure 3-2. Western blot Analysis of Daxx Protein Level in HEp2-H3-GFP Cells Expressing Control-siRNA or Daxx-siRNA. Note efficient depletion of Daxx using Daxx-specific siRNAs compared to control siRNAs.

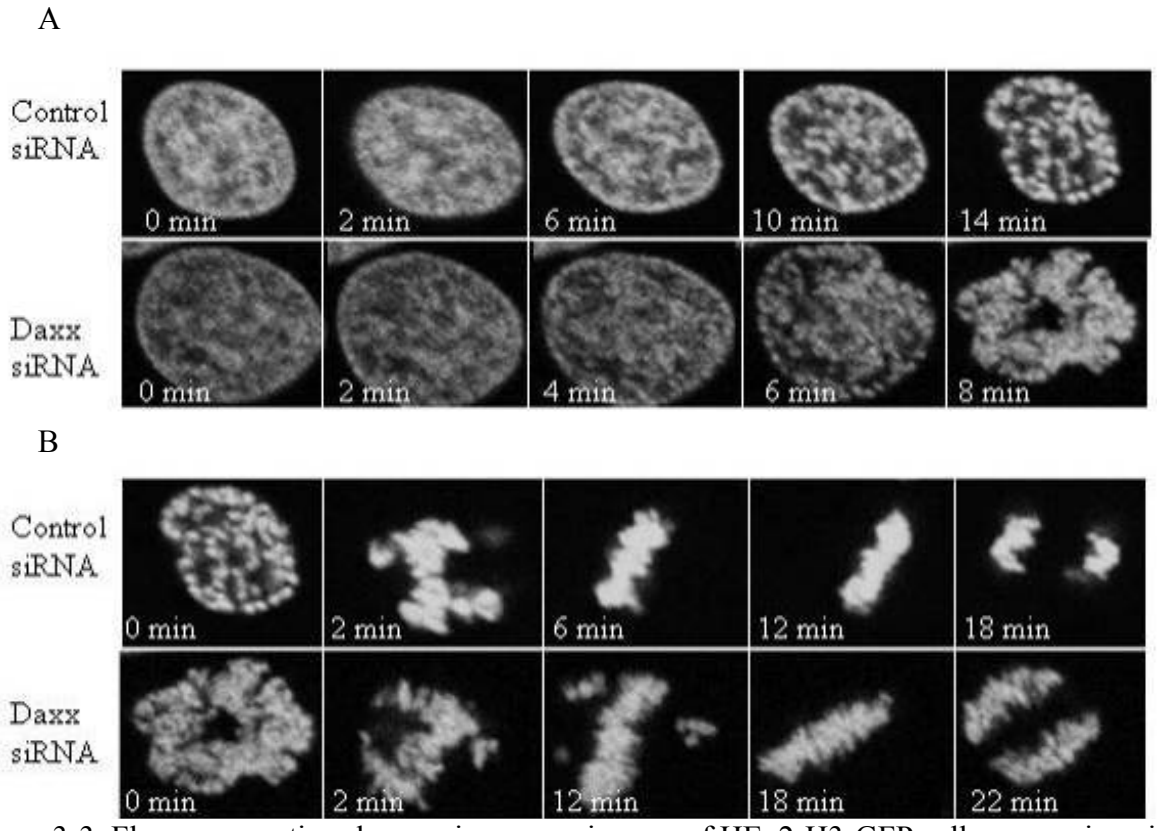


Figure 3-3. Fluorescence time-lapse microscopy images of HEp2-H3-GFP cells expressing either control-siRNA or Daxx-siRNA. A) Prophase progression in control and Daxx-depleted cell lines. B) Mitotic progression from nuclear envelope break-down (pro-metaphase) to chromosomal segregation (anaphase).

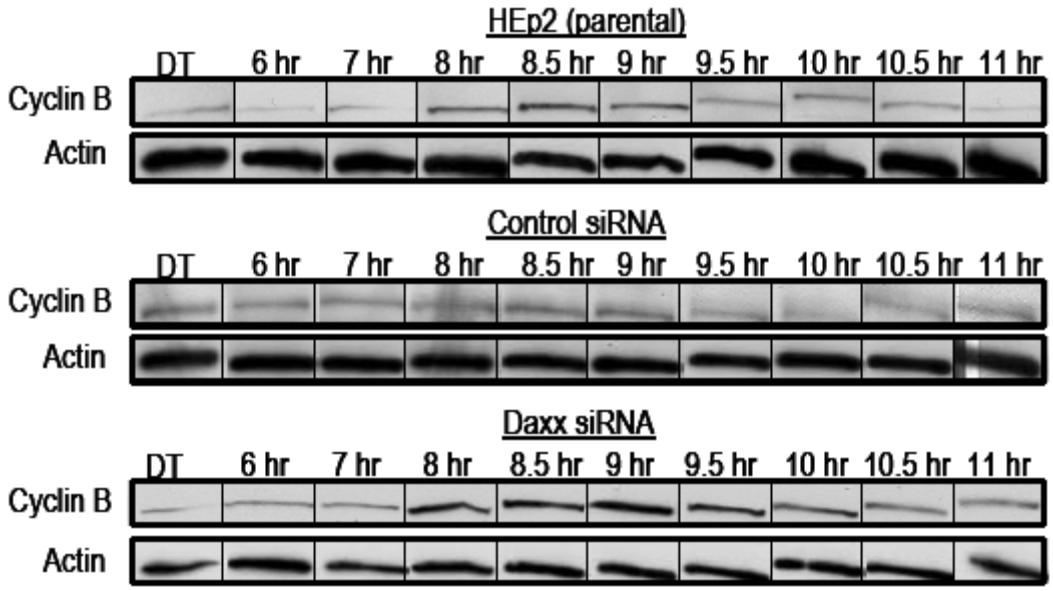


Figure 3-4. Daxx depletion stabilizes Cyclin B during mitosis. Mitotic progression of synchronized wild type (parental) HEp2 cells, control-siRNA and Daxx-siRNA cell lines. Cells were synchronized using a double thymidine block and released and probed for Cyclin B protein levels at the indicated time points.

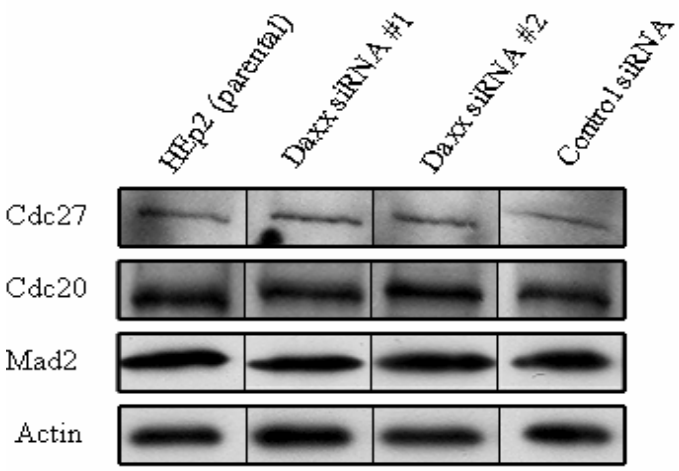


Figure 3-5. Western Blot Analysis of Mitotic Proteins in Wild type (Parental), Control-siRNA and Two independent Daxx-siRNA Cell Lines. Protein expression did not differ significantly in the presence or absence of Daxx.

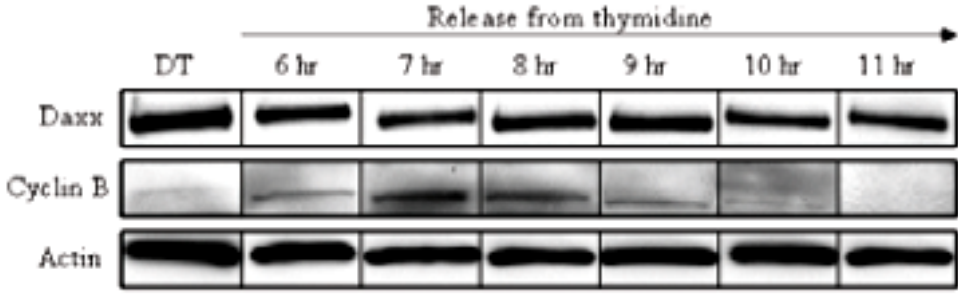
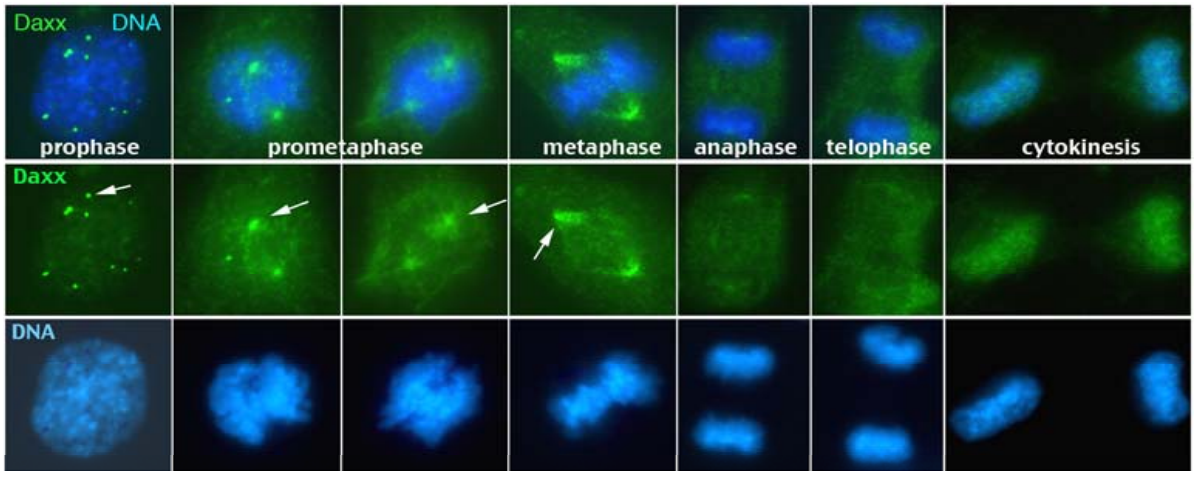


Figure 3-6. Western Blot Analysis of Daxx Protein Levels Throughout the Cell Cycle. HEp2 cells were synchronized in G1/S using a double thymidine block (DT) and then released from thymidine block for the indicated times. Daxx protein levels change insignificantly throughout each cell cycle stage.

A



B

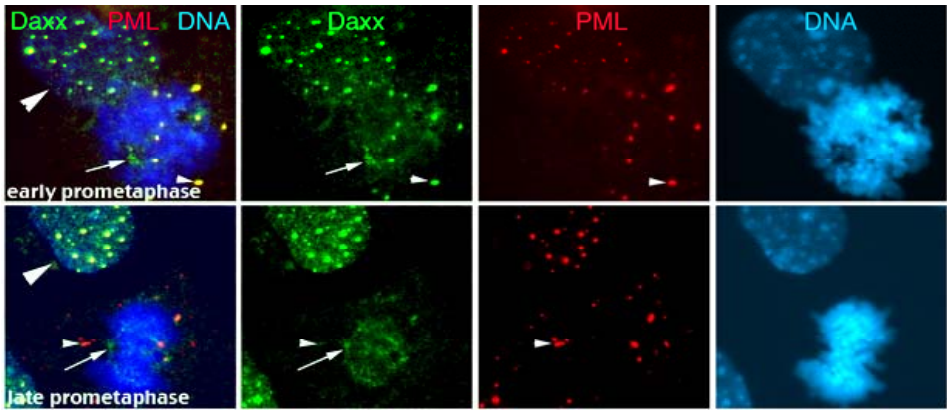


Figure 3-7. Dynamics of Daxx Localization in Mitotic MPEF Cells. A) Localization of Daxx in prophase (at PML), pro-metaphase (spindles), metaphase (spindles) and anaphase-cytokinesis (no association). B) Change in localization of Daxx during early pro-metaphase (top picture) from PML bodies to the forming spindle apparatus. By late pro-metaphase (bottom picture), the majority of Daxx is localized to the spindle apparatus.

CHAPTER 4
DAXX IS A TRIGGER OF CELLULAR TAXOL RESPONSE

Introduction

The mitotic spindle checkpoint is a very fluid and dynamic apparatus designed to monitor microtubule-kinetochore attachment and proper microtubule tension. In the event of an error occurring during pro-metaphase and metaphase, the spindle checkpoint will elicit a "wait" signal that stalls chromosomal segregation and anaphase onset until errors are corrected. Microtubule-inhibiting drugs such as taxol and nocodazole (among others) can initiate a prolonged "wait" signal that if uncorrected, initiates micronucleation (mitotic catastrophe) and cell death (Jiang et al., 2006; Mansilla et al., 2006a; Mansilla et al., 2006b; Ricci and Zong, 2006). Thus, sub-populations of cancer cells with deranged regulation of mitotic spindle checkpoint proteins and other associated factors can respond differently to these compounds offering a therapeutic molecular target for treating cancers. However, in most instances, inactivation or down-regulation of mitotic checkpoint proteins leads to increased sensitivity to microtubule inhibiting drugs. In cases of BubR1 or Mad2 depletion, cells treated with taxol respond in a much stronger and robust way: the number of cells arrested in mitosis dramatically decreased and cell death was more rapid (Sudo et al., 2004). Cells lacking Mad1, an important factor involved in the assembly of the spindle checkpoint, also exhibit increased sensitivity to taxol, however cells treated with nocodazole became more resistant (Kienitz et al., 2005). Thus, identification of novel regulators of taxol sensitivity—which may alter cellular response to these drugs by increasing resistance—is required.

Discussion and Results

Compounds that affect the mitotic spindle apparatus, including microtubule inhibitors such as paclitaxel (taxol) and nocodazole, are known to differentially affect cells that lack one or more mitotic spindle associated proteins compared to wild type cells. To further explore the potential role(s) of Daxx in mitosis and cell division, the microtubule inhibitor taxol, which is a microtubule hyper-stabilizing compound and the tubulin-destabilizing drug nocodazole were used to examine response of $Daxx^{+/+}$ and $Daxx^{-/-}$ MEFs. A very striking and divergent response was observed between $Daxx^{+/+}$ and $Daxx^{-/-}$ mouse fibroblasts treated with taxol and nocodazole (Figure 4-1), which was not recapitulated when cells were treated with drugs such as roscovitine, adriamycin and etoposide (data not shown). The number of mitotic cells in $Daxx^{+/+}$ MEFs was much lower compared to $Daxx^{-/-}$ MEFs. Conversely, the occurrence of micronucleated cells in $Daxx^{+/+}$ mouse fibroblasts was much higher in comparison to Daxx-deficient cells. This evidence implies a Daxx-specific function which is inhibited when cells are treated with taxol or nocodazole. The decrease in micronuclei and elevated mitotic index also correlated with cell survival in Daxx deficient cells, producing 90% of colonies after 24h of taxol treatment compared to untreated control, while the survival rate of $Daxx^{+/+}$ cells was only 15%--approximately 6 times lower compared to $Daxx^{-/-}$ cells (Figure 4-1). Given the similar time of cell cycle progression for both cell lines (not shown), the difference in colony formation most likely reflects a differential survival rate of cells upon drug exposure.

Given that taxol is a very potent chemotherapy agent used to treat breast cancers and other malignancies, the role of taxol-induced cell death in breast cancer cells was examined with how this may correlate with the level of protein Daxx. High heterogeneity of Daxx protein level among breast cancer cell lines was observed (Figure 4-2, normalized on actin). Daxx is a nuclear protein with obvious ND10/PML body association in interphase cells (Ishov et al., 1999); despite

high variety in Daxx protein level, intracellular distribution of Daxx is similar in all breast cancer cell lines tested, showing Daxx co-localizing with PML (not shown). To study a correlation between Daxx and cellular response to paclitaxel, cell lines with extreme level of Daxx were chosen: T47D (low level of Daxx, Daxx/actin = 1.0) and MDA MB 468 (high level of Daxx, Daxx/actin = 14.0) and tested for paclitaxel induced cell death measured by colony formation assay. Increased survival of T47D cells was observed compared to MDA MB 468 cells (Figure 4-2). 24h of treatment reduces the survival of MDA MB 468 cells almost three fold, and at 48h of treatment very few colonies formed. 24h treatment had almost no effect on T47D cell survival rate and 48h treatment reduced the number of colonies only by 30%. Thus, low level of Daxx correlates with increased resistance to paclitaxel treatment in these breast cancer cell lines.

To address the mechanism of cell death and differential survival of breast cancer cell lines T47D and MDA MB 468 upon paclitaxel treatment, morphological changes that occurred with nuclei were observed at different times of drug addition, which is effective means for discriminating between apoptosis and micronucleation. Cells were categorized based on the nuclear morphology (Figure 4-2). In mock-treated conditions (control, Figure 4-3) the majority of cells were in interphase. The rate of accumulation in mitosis at 12h is similar for both cell lines (Figure 4-3) that reflects almost an identical time of cell cycle progression. Already at 12h of treatment, 27% of micronucleated cells appear in MDA MB 468 cells and reaches 60% and 79% at 24h and 36h correspondingly (Figure 4-3, top graph), while only small portion of cells (20 and 8%) remains blocked in mitosis (Figure 4-3, middle graph). An insignificant number of T47D cells are micronucleated at 12h and 24h, reaching only 22% by 36h of treatment; most cells remain blocked in pro-metaphase during the course of treatment. The level of apoptosis in both cell lines was negligible, reaching a maximum of 2% and 7% correspondingly in T47D and

MDA MB 468 after 36h of treatment (Figure 4-3, bottom graph); thus, apoptosis is not the main mechanism of cell death for these cell lines at this drug concentration. Indeed, the process of apoptosis may occur as a secondary event or be more prominent (and is sometimes observed) in larger paclitaxel concentrations—but this may be outside of any clinical relevance (Hernandez-Vargas et al., 2006; Wang et al., 1999). Furthermore, upon exposure of MDA MB 468 and T47D cells to increased paclitaxel concentrations (100 nM and greater) sizeable increase in apoptotic levels or change in mitotic index/micronucleation could not be detected (Figure 4-4). This is in contrast to some reports showing that larger paclitaxel concentrations induce a more prominent mitotic checkpoint arrest and hence a stronger mitotic block in other cell lines (Giannakakou et al., 2001; Ikui et al., 2005). Similar results were observed by FACS analysis (Figure 4-5): the majority of T47D cells accumulate in G2/M at 24h and 36h of treatment, while MDA MB 468 produce an extensive sub-G1 population. Unfortunately, FACS does not allow discrimination between apoptotic and micronucleated cells as clearly as microscopic analysis because both types of cell death result in fragmentation of the nucleus (recognized as a sub-G1 population by FACS and thus counted together). Therefore, microscopy was the most useful approach to determine type of cell death after paclitaxel treatment. Thus, a major difference in paclitaxel response between these cell lines is the ability to maintain prometaphase block—which is extended in T47D, but is brief in MDA MB 468 and is followed by micronucleation.

Daxx was recently shown to interact with and regulate stability of p53, one of the key players in cell growth arrest and apoptosis (Tang et al., 2006; Zhao et al., 2004). Moreover, Daxx seems to differentially regulate p53 dependent transcription under DNA damage conditions in transient transfection assay, thus affecting the balance between cell cycle arrest and apoptosis (Gostissa et al., 2004). Stability of p53 and p53-dependent transcription regulation, however, were unaffected

in primary human fibroblasts depleted of Daxx and seems to be influenced instead by the JNK pathway during UV and H_2O_2 treatment (Khelifi et al., 2005). Both T47D and MDA-MB 468 express mutant, transcriptionally inactive p53 (Concin et al., 2003); thus, differences in paclitaxel response between these cell lines are most likely p53-independent.

To further confirm Daxx dependent cell survival upon paclitaxel treatment, Daxx protein levels were depleted in MDA MB 468 breast cancer cells, in HEp2 human epithelial carcinoma cells (Figure 4-6) and in human fibroblast cell line WI38 by stable expression of anti-Daxx siRNA (data not shown). In the case of both anti-Daxx siRNA, we observed a marked decrease and slower rate of micronucleation and an increase in mitotic index (Figure 4-6). In all cases, the levels of apoptosis throughout this ongoing process were negligible (data not shown). Importantly, the decline in micronucleation and increase of mitotic index observed in anti-Daxx siRNA cell lines correlated with an increased survival of cells under colony formation conditions (Figure 4-6). Thus, depletion of Daxx by siRNAs targeted against Daxx message in a variety of human cell lines reproduces the original finding that level of Daxx is critical for paclitaxel response.

The combination of these data allows the proposition of a model in which cells follow one of two paths in response to taxol and which is dependent on Daxx protein level: Daxx positive and taxol-sensitive cells will block in mitosis only transiently, followed by micronucleation, while Daxx deficient and taxol-resistant cells have prolonged mitotic block and continue proliferation after drug decay and microtubule dynamics restoration, thus surviving chemotherapy. This model emphasizes Daxx as a trigger for cellular taxol response—particularly in apoptosis-reluctant cells; cells lacking a functional Daxx protein display an increased resistance to taxane exposure.

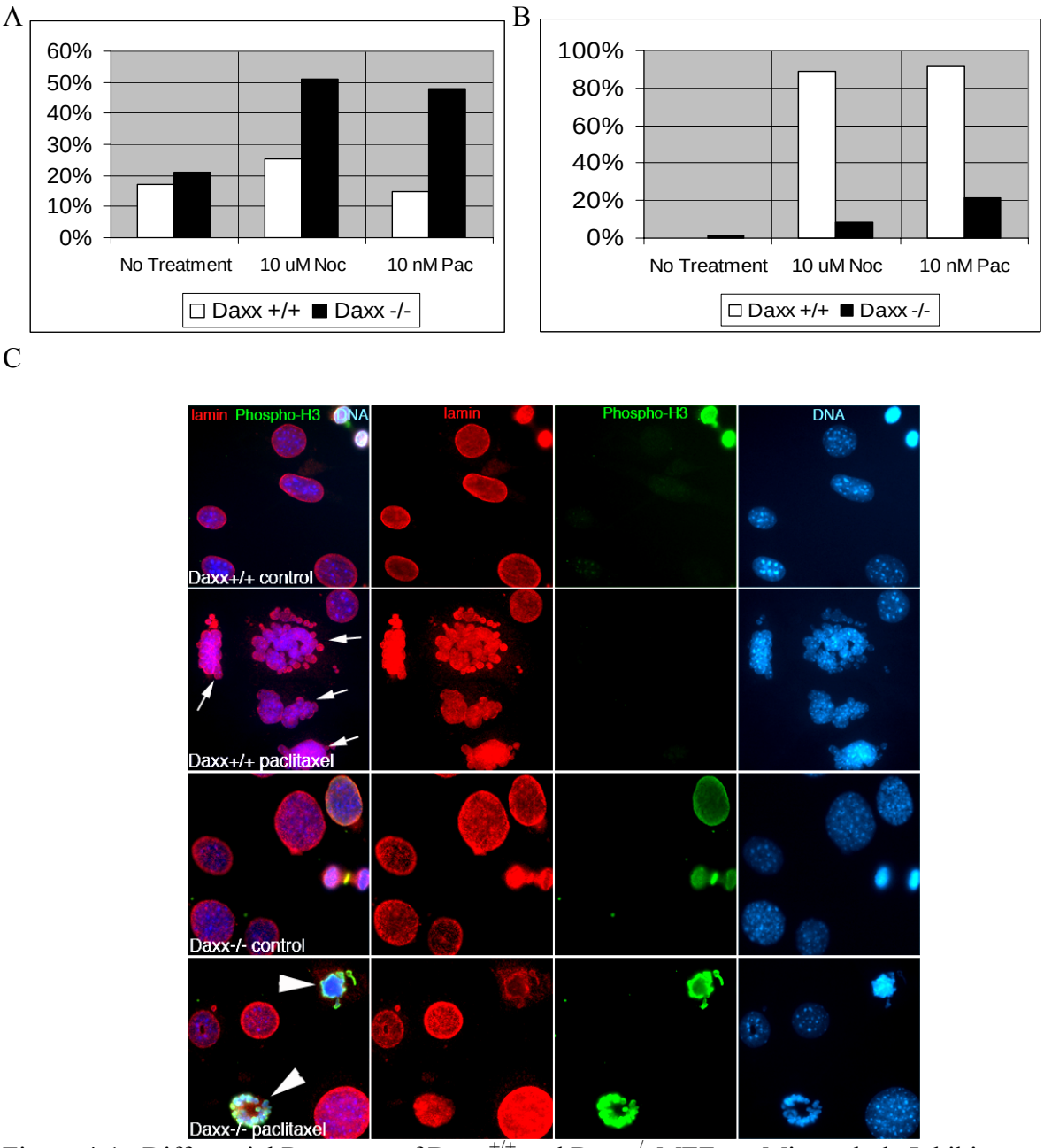


Figure 4-1. Differential Response of Daxx$^{+/+}$ and Daxx$^{-/-}$ MEFs to Microtubule Inhibitors Nocodazole and Paclitaxel. A) Mitotic index of MEFs treated with 10 M nocodazole or 10 nM paclitaxel for 24 hrs. Cells were fixed and stained with phospho-H3 antibody to characterize mitosis. B) Corresponding percentage of micronuclei formation in cells treated with nocodazole or paclitaxel for 24 hrs. C) Immunostaining of Daxx$^{+/+}$ and Daxx$^{-/-}$ MEFs treated with paclitaxel using mitotic markers phospho-H3 and lamin. Note occurrence of mitotic cells (big arrowheads) in Daxx$^{-/-}$ cells and micronuclei (small arrows) in Daxx$^{+/+}$ cells.

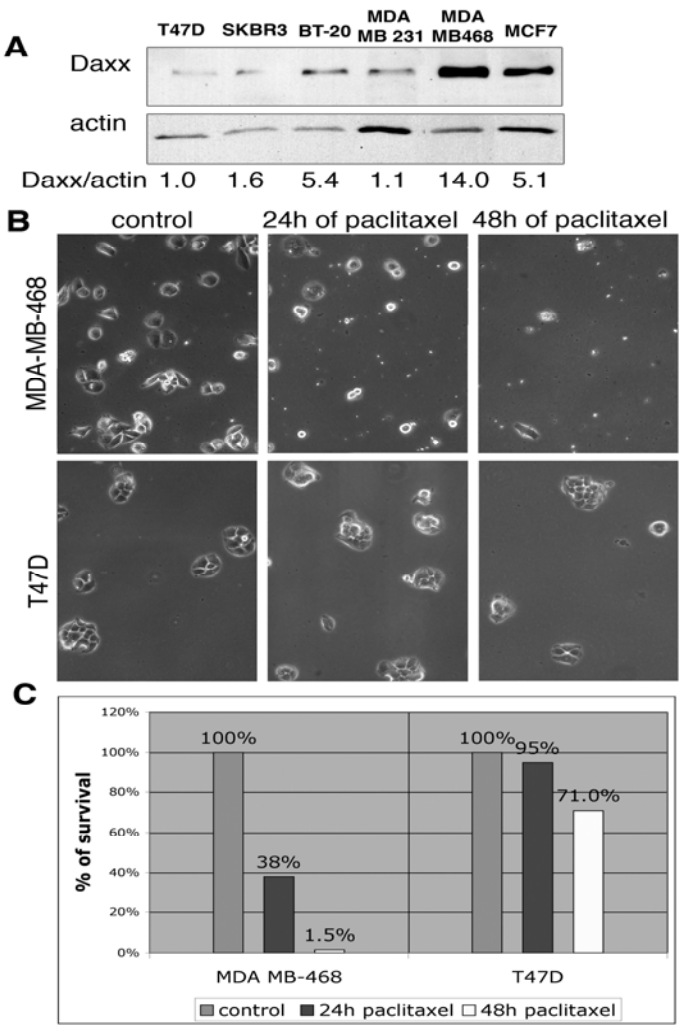


Figure 4-2. Colony Formation of Breast Cancer Cells After Paclitaxel Treatment is Daxx-Dependent. A) differential Daxx expression among breast cancer cell lines. Daxx accumulation normalized by actin (bottom). B) & C): MDA MB 468 (high Daxx) and T47D (low Daxx) were treated with 10 nM paclitaxel for 24h or 48h. Colonies were fixed and stained with crystal violet and calculated 5 days after drug withdrawal. Note differential taxol response (% of survival) between MDA MB 468 and T47D.

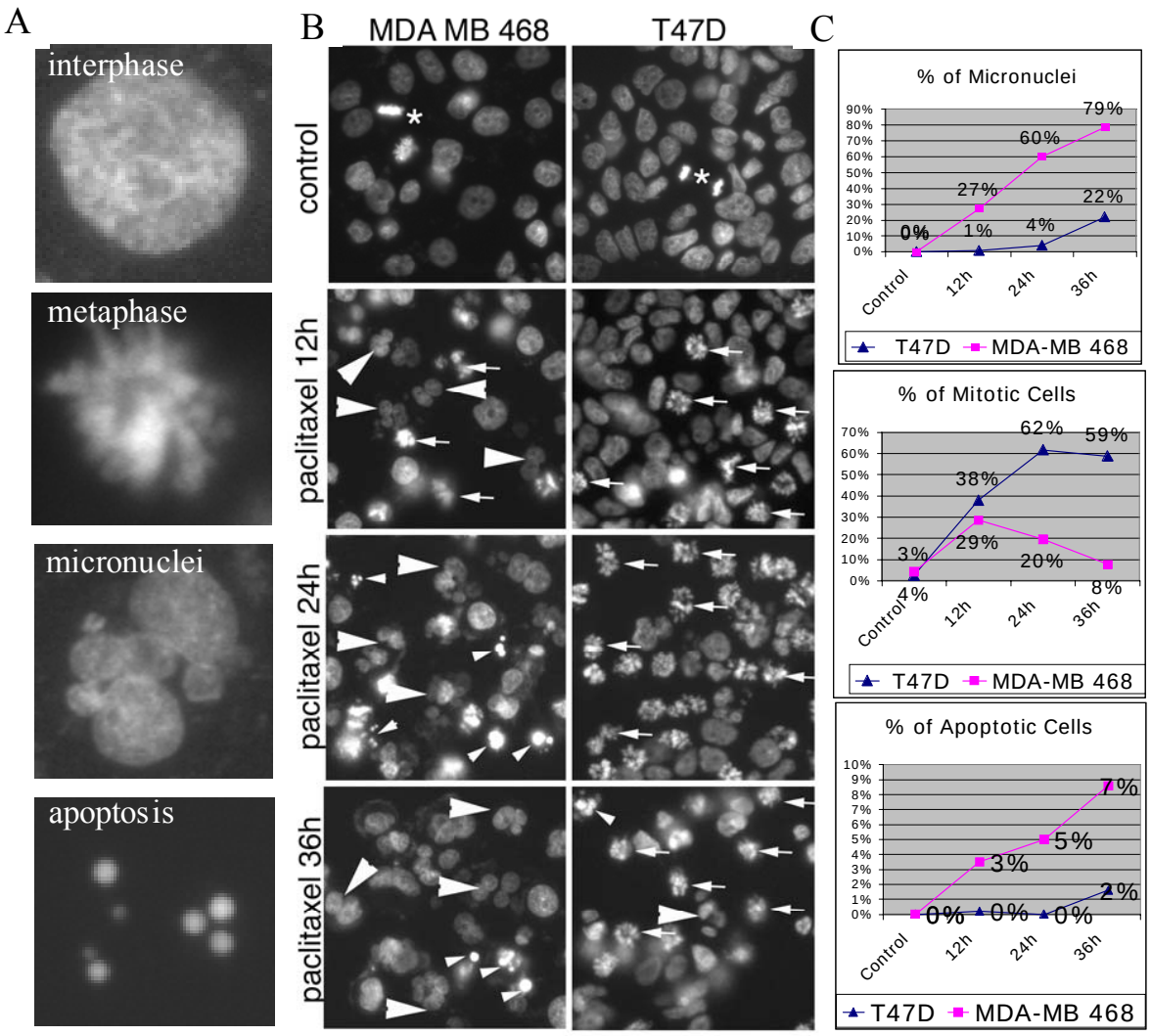
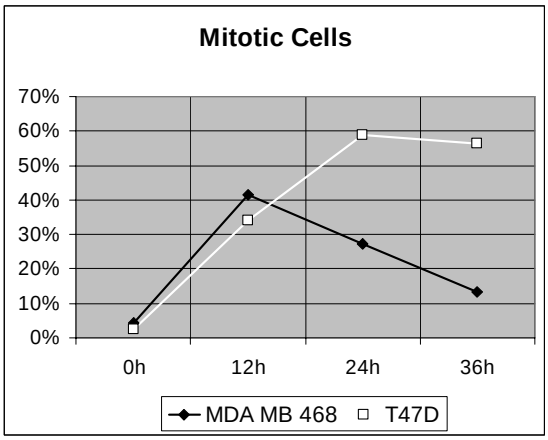
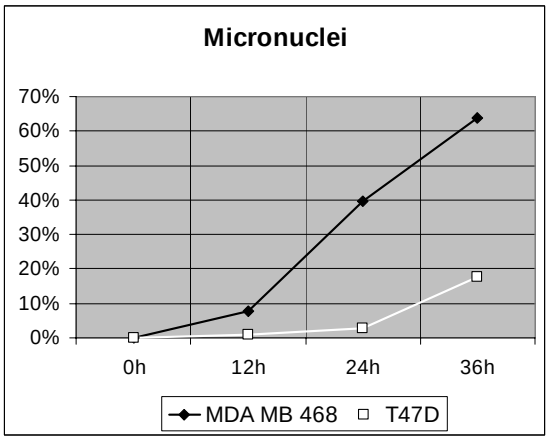


Figure 4-3. Response to Paclitaxel Treatment in Breast Cancer Cell Lines with Different Daxx Level. MDA MB 468 (high Daxx) and T47D (low Daxx) were treated with 10 nM paclitaxel for 12h, 24h or 36h or mock-treated (control). DNA was stained with HOECHST 33342. A) Cells were categorized as interphase, mitotic, micronucleated, and apoptotic based on the nuclear morphology. B) Mitotic cells in control: asterisks; micronucleated cells: big arrowheads; pro-metaphase cells: arrows; apoptotic cells: small arrowheads. While micronuclei appear in MDA MB 468 already after 12h of treatment, majority of T47D cells remain in pro-metaphase after 36h of treatment. C) Relative accumulation of mitotic cells, micronucleated and apoptotic cells in MDA MB 468 and T47D cells. The majority of MDA MB 468 cells execute micronucleation, while T47D are accumulated in pro-metaphase. For each time point one thousand cells were counted.

A



B



C

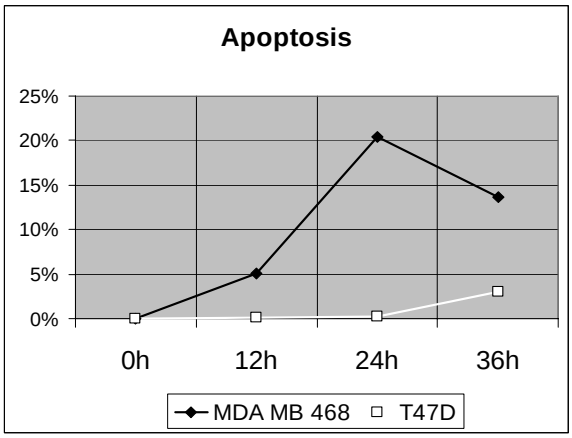


Figure 4-4. Response of MDA MB 468 and T47D Breast Cancer Cell Lines to Increased Concentration of Paclitaxel. MDA MB 468 and T47D cell lines were treated with 100 nM paclitaxel for 12h, 24h and 36h or mock treated (control). A) DNA was stained with HOECHST 33342 and cells were characterized as being mitotic, B) micronuclei or C) apoptotic, based on nuclear morphology. Cellular response to paclitaxel between these two cell lines remained relatively unchanged despite increased paclitaxel concentrations. Note: apoptosis does not significantly increase with elevated concentrations of paclitaxel.

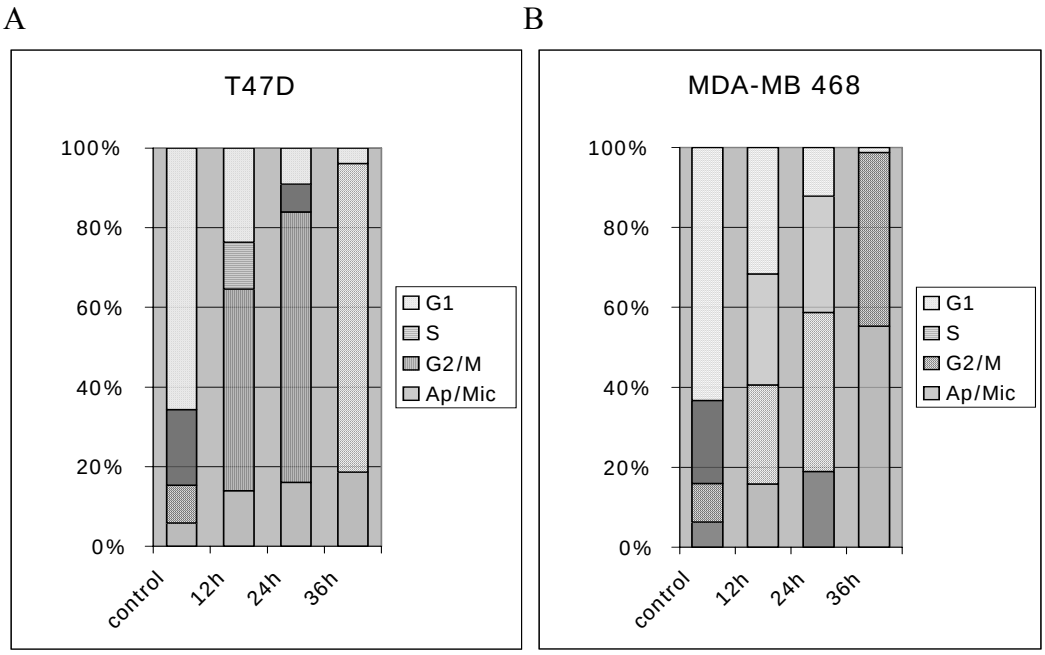
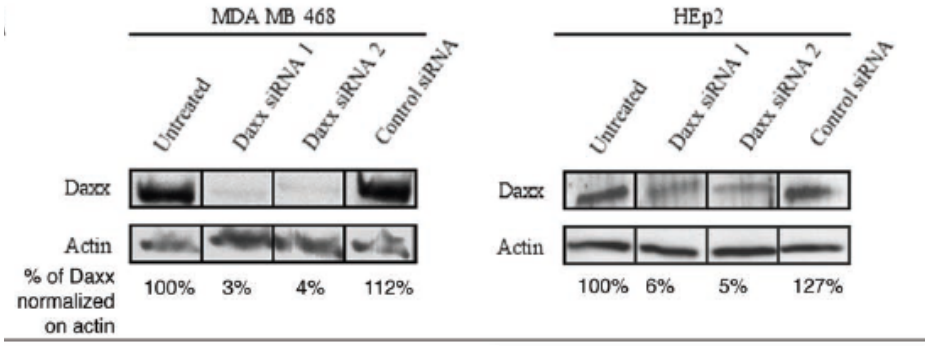
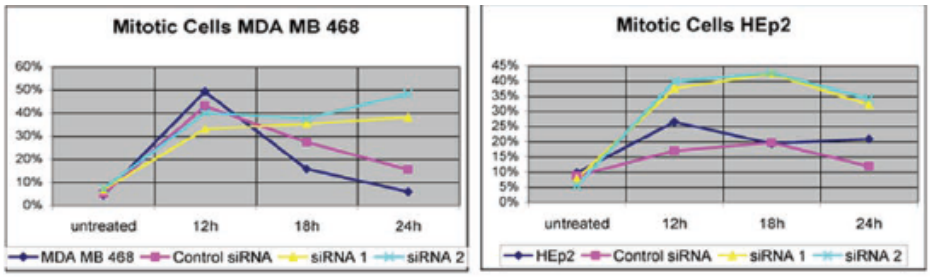


Figure 4-5. FACS Analysis of Cell Cycle Distribution after Paclitaxel Treatment. A) T47D and B) MDA MB 468 cells were treated with 10nM paclitaxel for the indicated time and sorted as G1 phase, S phase, G2/M phase, and apoptotic + micronucleated (Ap/Mic). While the majority of T47D cells accumulate in G2/M after 36h of treatment, MDA-MB 468 cells are mostly apoptotic + micronucleated at this time point. Unfortunately, FACS does not allow discrimination between apoptotic and micronucleated cells.

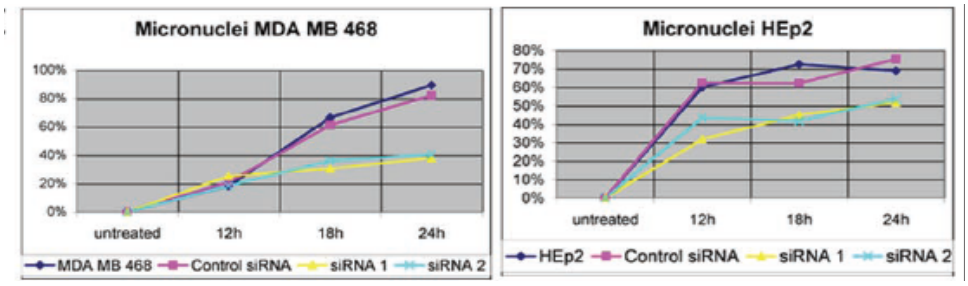
A



B



C



D

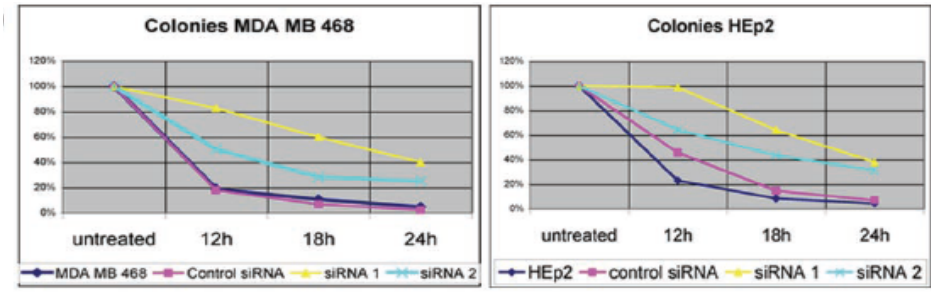


Figure 4-6. Paclitaxel Response is Daxx-Dependent. A) Western blot analysis of Daxx depletion in MDA MB 468 and HEp2 cells. B) Mitotic index of parental MDA MB 468 (left) and HEp2 cells (right) expressing two independent anti-Daxx siRNAs or control siRNA which were synchronized via double thymidine block and then released and treated with 10 nM paclitaxel for 12, 18 and 24 hrs. C) Same as (B) except micronucleated cells are shown. D) Reduction of Daxx increases cell survival during paclitaxel treatment. Cells treated in (B) and (C) were set up for colony formation assay and allowed to grow for 5 days. Colonies were then counted after fixation and staining with crystal violet. Apoptosis, as a result of paclitaxel exposure, was negligible across all of these different cell lines (data not shown).

CHAPTER 5
DAXX INTERACTS WITH RAS-ASSOCIATION DOMAIN FAMILY 1 (RASSF1) WHICH COOPERATE IN CELLULAR TAXOL RESPONSE

Introduction

Several lines of evidence suggest Daxx is important for proper timing of mitotic progression and that paclitaxel (taxol) resistance can be dependent on the level of Daxx protein in cells. Firstly, cells generated from Daxx-deficient embryos exhibit genomic instability while tissue sectioning of E9.5 Daxx-deficient mouse embryos shows an accumulation of cells in early mitosis (specifically pro-metaphase). Targeted depletion of Daxx also results in alteration of mitotic progression during prophase and the pro-metaphase to anaphase transitions. Additionally, proper degradation of Cyclin B in synchronized Daxx-depleted cells is altered. Importantly, cells that are deficient or depleted of Daxx was proven to provide resistance to the microtubule inhibiting drug taxol by arresting cells in mitosis; cells with a functional Daxx protein have a shortened mitotic arrest resulting in micronucleation and cell death (Lindsay et al., 2007).

Discussion and Results

To further investigate Daxx mitotic function and to study the potential mechanism by which Daxx regulates taxol response during mitosis a search for Daxx-interacting proteins using Daxx as "bait" in a yeast two-hybrid screen was performed. Several yeast two-hybrid screens have been used in the past using Daxx as both "bait" and "prey." The majority of these screens have used, or have identified through their screens, the carboxyl-terminal region of Daxx (corresponding with PML- and Fas-interacting domains) which also contains a region of Daxx

known to interact with sumoylated proteins (Pluta et al., 1998; Yang et al., 1997). Since yeast themselves frequently use sumoylation for protein-protein interactions, this can potentially explain the frequent occurrence of Daxx-false positives from yeast two hybrid screens (Michaelson, 2000). For this analysis, the amino-terminal region of Daxx was used which has previously not been adapted for yeast two-hybrid screens. This novel approach identified several proteins with functional implications in the regulation of cell division and mitosis. Sequence analysis of two particularly strong interaction clones revealed homology with amino acids 5-270 and 30-270 of mouse RAS associated domain family 1 splice form C (Rassf1C) (Figure 5-1). Retransformation and β-gal reporter assay confirmed the specificity of this interaction in yeast, pointing at the amino -terminus of Daxx as a potential region of Rassf1 interaction (Figure 5-1). DMAP1 (DNA methyltransferase associated protein 1) that was recently shown to interact with Daxx (Muromoto et al., 2004) was used as positive control in these experiments. Rassf1 is a highly conserved throughout species and its locus is frequently altered in human cancer. Among several alternative splice forms, Rassf1A and Rassf1C are the most abundantly expressed. Both Rassf1A and Rassf1C are tubulin-associated proteins which influence the overall stability and dynamics of microtubules (Liu et al., 2003). While Rassf1C function is relatively unknown, Rassf1A has recently been shown to regulate early mitosis progression, particularly during prometaphase (Song et al., 2004). In addition, Rassf1A over-expression leads to mitotic arrest and inhibition of colony growth (Liu et al., 2003).

$Daxx^{-/-}$ embryos showed an increased accumulation of cells in pro-metaphase, suggesting that Daxx could cooperate with Rassf1A function during pro-metaphase. In light of the known properties of both Rassf1A and Rassf1C on the influence of microtubules, the potential interplay of Daxx and Rassf1 binding could also partially explain alterations in sensitivity of Daxx-

depleted cells to taxol, a known microtubule-interfering compound. Interaction mapping between Daxx, Rassf1C and Rassf1A and deletion mutants was analyzed with proteins *in vitro* using a pull-down assay and by co-localization of GFP and RFP fusion proteins in cells (Figure 5-2). Interaction between Daxx and Rassf1 was tested by incubation of immobilized 6His-fusion Rassf1C with GST-Daxx and GST alone (Figure 5-2). Rassf1C associates only with GST-Daxx and does not associate with GST control that indicates specificity of Rassf1C-Daxx interaction. All truncation mutants of Daxx, including first 142 amino acids of protein, were co-purified with Rassf1C wt suggesting that region of Rassf1 interaction is located at the amino terminus of the Daxx protein, thus confirming yeast two-hybrid data (Figure 5-2). Co-localization was observed between Rassf1C-RFP and GFP-fused Daxx wt and Daxx amino terminal deletion mutants but not with a carboxyl terminus mutant of Daxx (amino acids 625-740), further pointing at the amino terminus of Daxx as the minimal Rassf1 interacting domain.

GFP-Daxx does not co-localize with RFP-Rassf1A wt or with a Rassf1A mutant that covers aa 120-340 which is homologous between Rassf1A and Rassf1C, but GFP-Daxx co-localizes with the first 50 amino acids of RFP-Rassf1C that are unique for this splice variant. Moreover, the first 142 amino acids of Daxx can also co-localize with this domain of Rassf1C. In conclusion, the interaction between Rassf1 and Daxx is facilitated by the first 50 unique amino acids of Rassf1C; in combination with a minimal clone purified in a yeast two-hybrid analysis (which encodes amino acids 30 to 270 of Rassf1C) the potential minimal region of interaction can likely be narrowed down to amino acids 30-50 of Rassf1C. Therefore, Daxx interacts with Rassf1C, but not Rassf1A.

Considering these data, it was hypothesized that Daxx may cooperate with Rassf1A functioning in mitosis via an interaction with Rassf1C. This model assumes that two alternative

spliced proteins, Rassf1A and Rassf1C, can interact with each other and have similar localization during mitosis or throughout the cell cycle. It was found that GST-Rassf1A could bind 6His-Rassf1C in a pull-down assay (Figure 5-2B). Furthermore, a Rassf1A mutant (aa 194-340) could still efficiently bind Rassf1C (Figure 5-2B). Additional evidence of interaction was obtained by co-localization of GFP- and RFP-fused Rassf1A/Rassf1C. Co-localization between Rassf1C and amino acids 194-258 of Rassf1A indicates smallest region of interaction. Fluorescence time-lapse microscopy of HEp2 cells expressing GFP-Rassf1A wt or GFP-Rassf1C wt also revealed similar localization throughout the cell cycle implying a potentially constant association of Rassf1A-Rassf1C proteins (data not shown). Thus, the minimal amino acids necessary for Rassf1A-Rassf1C interaction likely resides within the common Ras-Association (RA) domain shared by both splice forms of Rassf1, which confirms hetero-dimer formation of Rassf1A and Rassf1C and also opens the possibility of homo-dimer formation between each of these isoforms.

To confirm the endogenous interaction of Daxx and Rassf1, double immunofluorescent staining of Daxx and Rassf1C was performed in HEp2 cells where these two proteins were found to be distinctly separated during interphase (Figure 5-3) with Daxx staining relegated strictly to the nucleus (at PML bodies) and Rassf1C staining localized strictly to a microtubule-network pattern. This is in contrast to some reports suggesting Rassf1C is a nuclear protein that may interact with Daxx at PML (Kitagawa et al., 2006). To confirm the differential localization of Daxx and Rassf1 in cells, biochemical separation of HEp2 cells into nuclear and cytosolic fractions was performed as well as 3D-confocal imaging of transiently over-expressed GFP-Rassf1A, GFP-Rassf1C and GFP-Daxx (Figure 5-3 B & C). Daxx was found to be largely a nuclear associated protein while Rassf1A appeared strictly in the cytosolic fraction by biochemical separation. Three-dimensional confocal analysis of Daxx and Rassf1 cellular

localization showed Daxx to be a strictly nuclear associated protein, as expected. In stark contrast, both GFP-Rassf1A and GFP-Rassf1C displayed cytoplasmic distribution in a microtubule-like network with no GFP-fluorescence emanating from the nucleus. Thus, Daxx and Rassf1 are compartmentally separated proteins during interphase. Co-localization of endogenous Daxx and Rassf1, however, was observed in HEp2 cells progressing through mitosis—demonstrating the cell cycle regulated interaction between these proteins (Figure 5-4). Co-localization of Daxx and Rassf1 was observed beginning in pro-metaphase and metaphase, but by later stages of mitosis this association could not be detected.

To understand the cell cycle regulated interaction of Daxx and Rassf1 during mitosis and cellular paclitaxel response, stable expressing Rassf1A-siRNA was introduced into HEp2 cells which efficiently deplete Rassf1A protein level (Figure 5-5). While Rassf1A-depleted cells could be easily generated, over-expression of Rassf1A leads to cell toxicity and inhibition of cell proliferation independent of Rassf1A-functioning in cells. Therefore these studies focused primarily on protein-depleted cell lines. In combination with control and anti-Daxx siRNA HEp2 lines, anti-Rassf1A siRNA HEp2 cells were exposed to 10 nM paclitaxel for 6-18 hrs and then replated for colony formation assay (Figure 5-5). Strikingly, in the case of both Daxx- and Rassf1A-depleted cells, paclitaxel resistance was similar. Both cell lines exhibited a strong paclitaxel resistant phenotype with the majority of treated cells (75-80%) capable of dividing and forming colonies after removal of taxol. To gain a biochemical understanding of how mitotic cells respond to paclitaxel in the absence of Daxx or Rassf1A, control-, Daxx- and Rassf1A-depleted cells were synchronized using a double thymidine block and then released and exposed to taxol for 6-18 hrs and collected for Western-blot evaluation of Cyclin B protein levels (Figure 5-6). Wild type (parental) and control-siRNA cells revealed an accumulation of Cyclin B as

cells entered mitosis, but a marked drop-off in Cyclin B protein level ensued indicating cell exit from mitosis in the later stages of paclitaxel treatment. In contrast, Daxx- and Rassf1A-siRNA cells showed a similar accumulation of Cyclin B but these levels were maintained throughout the course of the experiment, indicating cells were still arrested in mitosis. Thus, Daxx and Rassf1A are necessary for efficient cellular response to paclitaxel which includes entry into and exit from mitosis during treatment.

Sustained Cyclin B protein levels in response to mitotic stresses like paclitaxel is an indication of prolonged spindle checkpoint activation (Musacchio and Salmon, 2007). During normal cellular response to paclitaxel, cells will transiently arrest in mitosis due to an activated spindle checkpoint but will exit mitosis by degrading mitotic substrates (i.e. Cyclin B) because taxol-generated errors (i.e. microtubular tension, unattached kinetechores) cannot be corrected. In the absence of Daxx or Rassf1A, cells remain in a prolonged mitotic block as evidenced by sustained Cyclin B protein levels. Many different regulatory proteins are involved in proper spindle checkpoint operation, including the Aurora kinases, a family of serine/threonine kinases that are highly conserved phylogenetically. Specifically, Aurora A and Aurora B are involved in the proper placement and localization of key mitotic checkpoint proteins (Ditchfield et al., 2003) and absence or depletion of Aurora kinases causes spindle checkpoint-override (Fu et al., 2007). Therapeutically, it would be advantageous to target Aurora kinases in tumors because Aurora A and Aurora B are frequently up-regulated in cancers (Keen and Taylor, 2004). As a result, several Aurora kinase inhibitors are in phase I & II clinical trials to evaluate their efficacy as chemotherapeutic agents (Agnese et al., 2007; Keen and Taylor, 2004). Clinical strategies for enhancing paclitaxel response are continuously being studied and developed and one potential method involves the use of taxol in combination with Aurora kinase inhibitors (Malumbres,

2006). Current compounds, including ZM447439, hesperadin and VX680, have been engineered to target the ATP binding site of Aurora kinases which abolishes their kinase activity. In addition to paclitaxel, using these compounds has been shown in cell-based assays to alter taxol response, even when spindle checkpoint proteins were absent (Morrow et al., 2005). Thus, the potentials of abrogating taxol resistance in combination with other compounds which target the mitotic spindle checkpoint are promising.

In order to determine if Daxx- and Rassf1A-mediated taxol response can be altered by inhibition of Aurora kinase activity, control-, Daxx- and Rassf1A-depleted HEp2 cells were treated with taxol in combination with two independent Aurora kinase inhibitors (Figure 5-7). Compounds used in this study were ZM447439, which targets Aurora A and Aurora B kinase activity and Aurora kinase inhibitor III, which targets Aurora A kinase activity. After synchronization with a double thymidine block and a six hour release, control and experimental cell lines were exposed to paclitaxel alone or paclitaxel in combination with ZM447439 or Aurora kinase inhibitor III for a period of six hours. After completion of drug exposure, cells were then replated for colony formation assay. Treatment of Daxx- or Rassf1A-depleted HEp2 cells with paclitaxel alone typically resulted in a very robust taxol resistance, as evidenced by the 75-80% survival rate of these cell lines compared to only 46% of control cells (Figure 5-7). Strikingly, however, taxol resistance was abolished in Daxx- or Rassf1A-depleted HEp2 cells when treated in combination with ZM447439 or Aurora kinase inhibitor III, resulting in comparable cell survival with control cells (Daxx-siRNA 31%-42%, Rassf1A siRNA 38%-40% and control siRNA 29%-37%). Thus, in the absence of Daxx or Rassf1A, functional inactivation of the mitotic spindle checkpoint using Aurora kinase inhibitors can change cellular taxol response and abolish resistance.

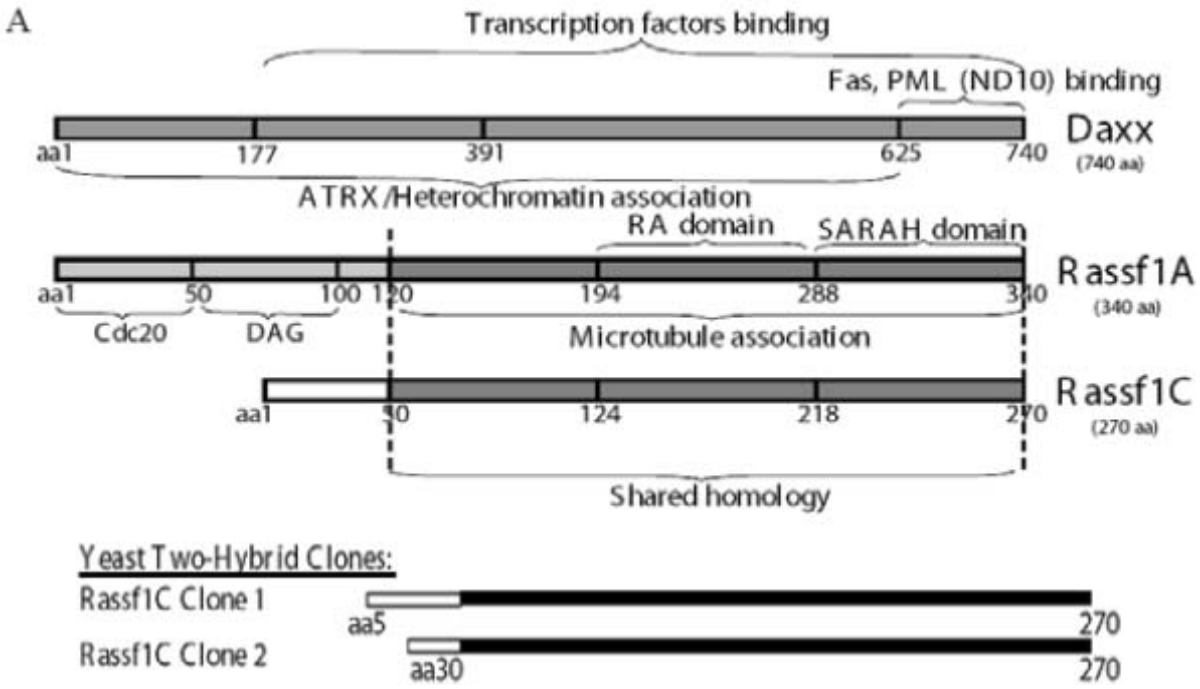


Figure 5-1. Daxx Interacts with Tumor Suppressor Rassf1 in Yeast. A) Schematics of Daxx and Rassf1 isoforms Rassf1A and Rassf1C along with mapping of clones identified through yeast two hybrid screening (Rassf1C clone 1 corresponding to amino acids (aa) 5-270 of mouse Rassf1C, Rassf1C clone 2 corresponding to aa 30-270). Homology between Rassf1A and Rassf1C is also shown. B) Retransformation assay of Daxx and Rassf1C constructs. 1 = Rassf1C + pGBDC1 (empty vector), 2 = Rassf1C + Daxx wt, 3 = Rassf1C + Daxx C term, 4 = Rassf1C + Daxx ΔC. C) β–Gal reporter assay measuring strength of interaction between individual Rassf1 clones and Daxx wt and deletion mutants. Strong interaction between Rassf1 clone 2 is observed. DMAP (positive control for Daxx interaction) was used as an evaluation for strength of interaction in this system.

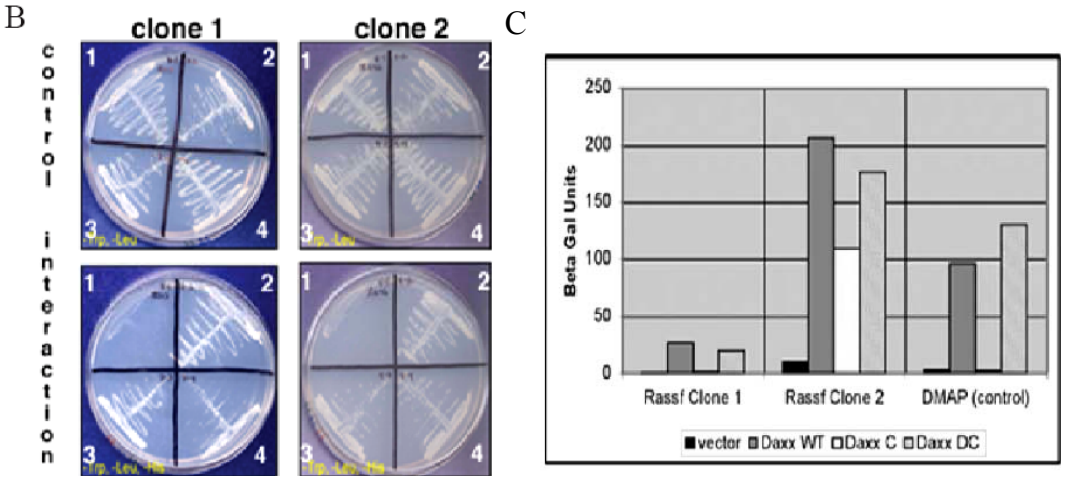
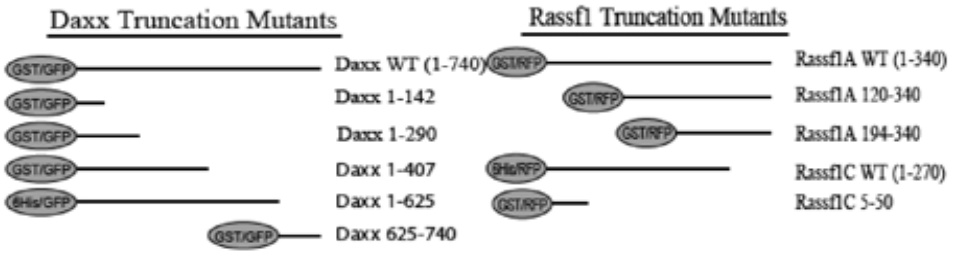


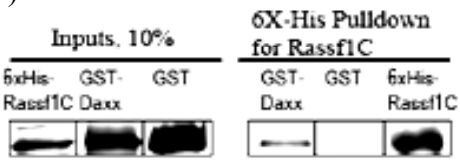
Figure 5-1. Continued.

A

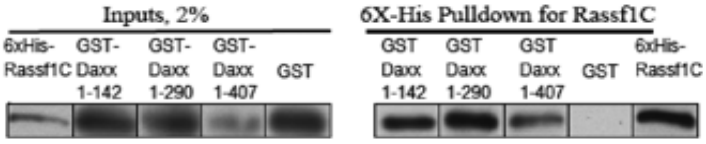


B

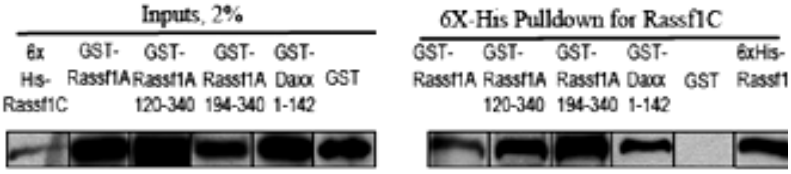
(i)



(ii)



(iii)

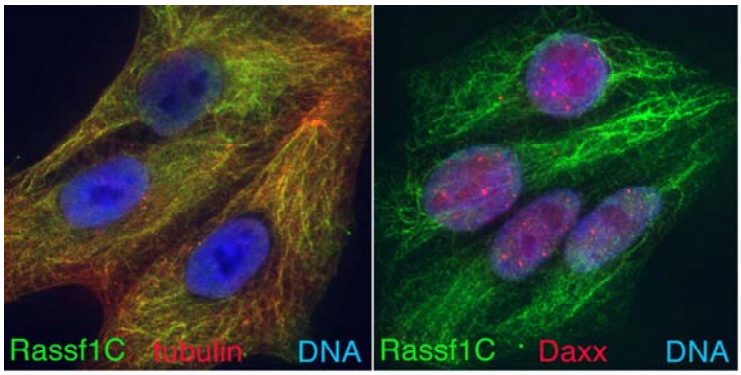


C

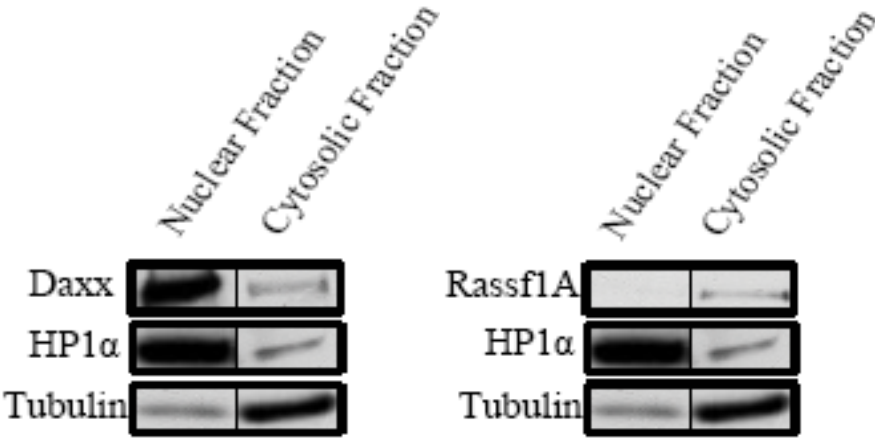
Results of: IF / Pulldown (+ or -)	Daxx 1-740	Daxx 1-142	Daxx 1-290	Daxx 1-407	Daxx 1-625	Daxx 625-740
Rassf1A 1-340	-/ND	-/ND	-/ND	-/ND	-/ND	-/ND
Rassf1A 120-340	-/ND	-/ND	-/ND	-/ND	-/ND	-/ND
Rassf1C 1-270	++/+	+++/+	++/+	+/+	+/ND	-/ND
Rassf1C 1-50	+/+	+++/+	++/+	+/+	+/ND	-/ND

Figure 5-2. Mapping of Daxx-Rassf1 Interaction. A) Diagram of human Daxx, Rassf1A and Rassf1C constructs used for mapping interaction by co-localization (GFP and RFP) and *in vitro* pull down assay (GST and 6His). B) (i) 6xHis pull down assay of GST-Daxx wt and 6xHis-Rassf1C wt. Immobilized 6xHis-Rassf1C wt was incubated with either GST or GST-Daxx wt. GST-Daxx wt but not GST alone binds 6xHis-Rassf1C wt. (ii) In similar experimental settings, all Daxx amino terminal constructs including aa 1-142 retain capacity to bind 6His-Rassf1C wt. (iii) 6His pull down assay of GST-Rassf1A wt and mutants using immobilized 6His-Rassf1C wt. GST-Rassf1A wt/ mutants, GST-Daxx 1-142 and GST alone were incubated with 6His-Rassf1C wt. GST-Daxx 1-142, GST-Rassf1A wt and mutants 120-340 and 194-340 bind to Rassf1C, while GST does not. C) Table summarizing interaction (+, –, or ND for not determined) tested by co-localization of GFP and RFP fusions or *in vitro* pull down assay (right in cell).

A



B



C

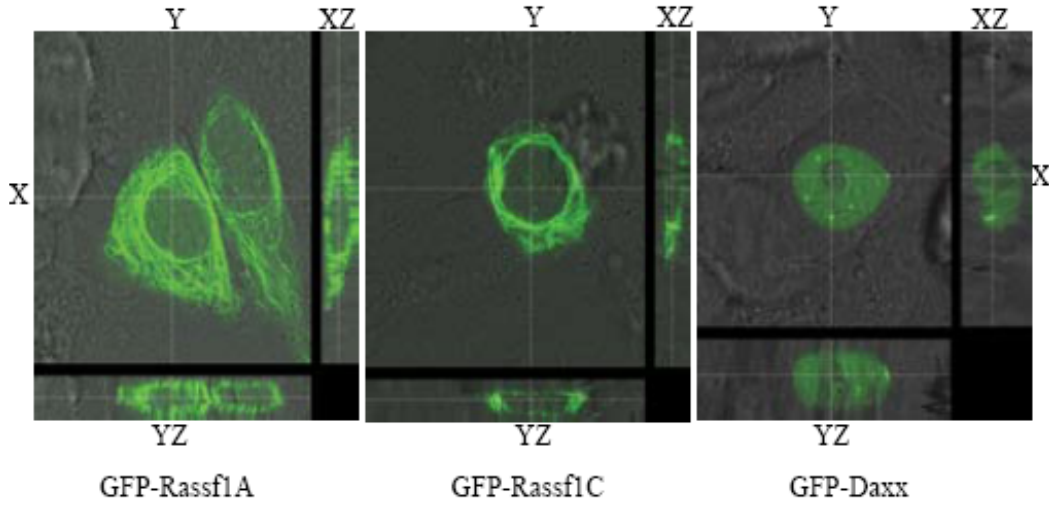
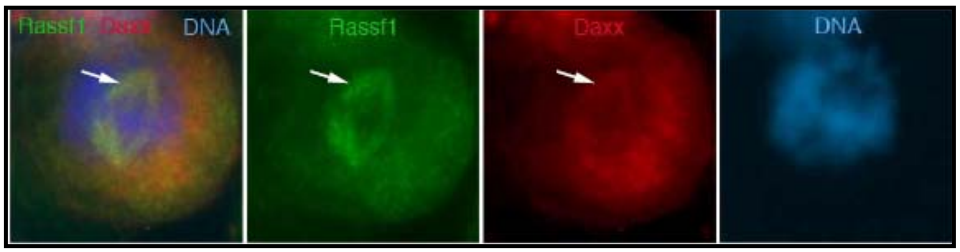
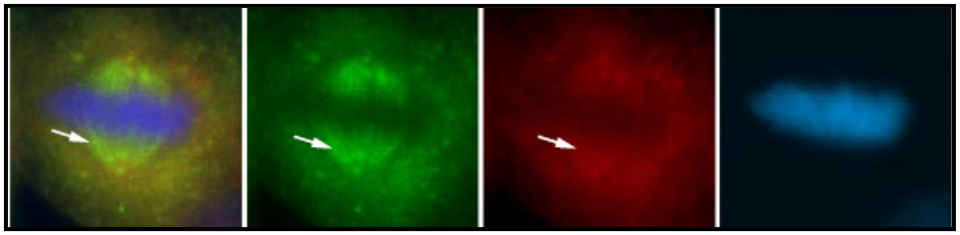


Figure 5-3. Cellular Distribution of Daxx and Rassf1 During Interphase. A) Immunostaining of endogenous Rassf1C and Daxx in interphase HEp2 cells. B) Biochemical separation of nuclear and cytosolic fractions from HEp2 cells. Note nuclear association of Daxx and cytosolic association of Rassf1A. C) 3D confocal imaging of transiently over-expressed GFP-Rassf1A (far left), GFP-Rassf1C (middle) and GFP-Daxx (far right). Note the compartmentally separated expression of Daxx and Rassf1.

A



B



C

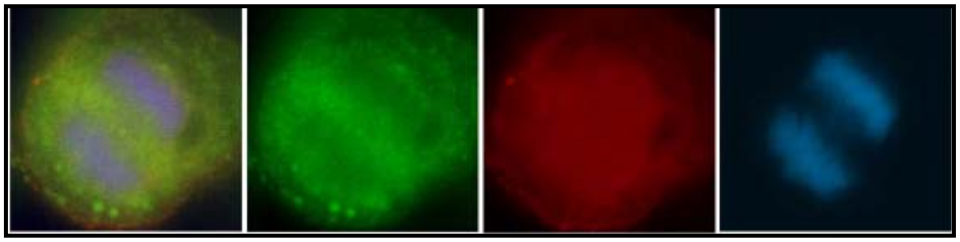
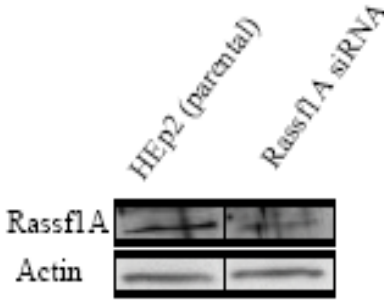


Figure 5-4. Co-localization of Endogenous Daxx and Rassf1 During Mitosis in HEp2 Cells. HEp2 cells were immunostained with monoclonal Daxx 514 antibody and polyclonal Rassf1 antibody which detects both endogenous Rassf1A and Rassf1C. Rassf1 and Daxx co-localized during pro-metaphase and metaphase A) & B). C) By anaphase and later stages of mitosis, this association is absent.

A



B

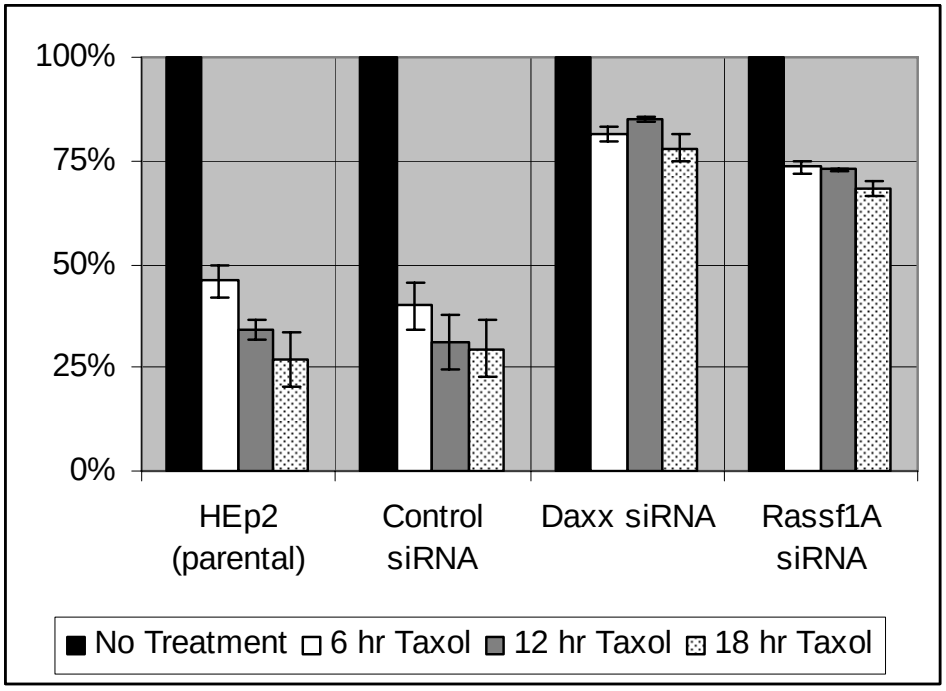


Figure 5-5. Depletion of Daxx or Rassf1A Desensitizes Cells to Paclitaxel. A) Stable expression of anti-Rassf1A siRNA in HEp2 cells. Note depletion of Rassf1A protein compared to parental cell lines. B) Percentage of colonies formed from parental, control, Daxx- and Rassf1A-depleted HEp2 cells which were synchronized using a double thymidine block and then released and exposed to paclitaxel for the indicated time periods (6, 12, 18hrs). After treatment, cells were replated for colony formation assay. Note increased survival (paclitaxel resistance) of Daxx- and Rassf1A-depleted cells compared to control and parental cell lines.

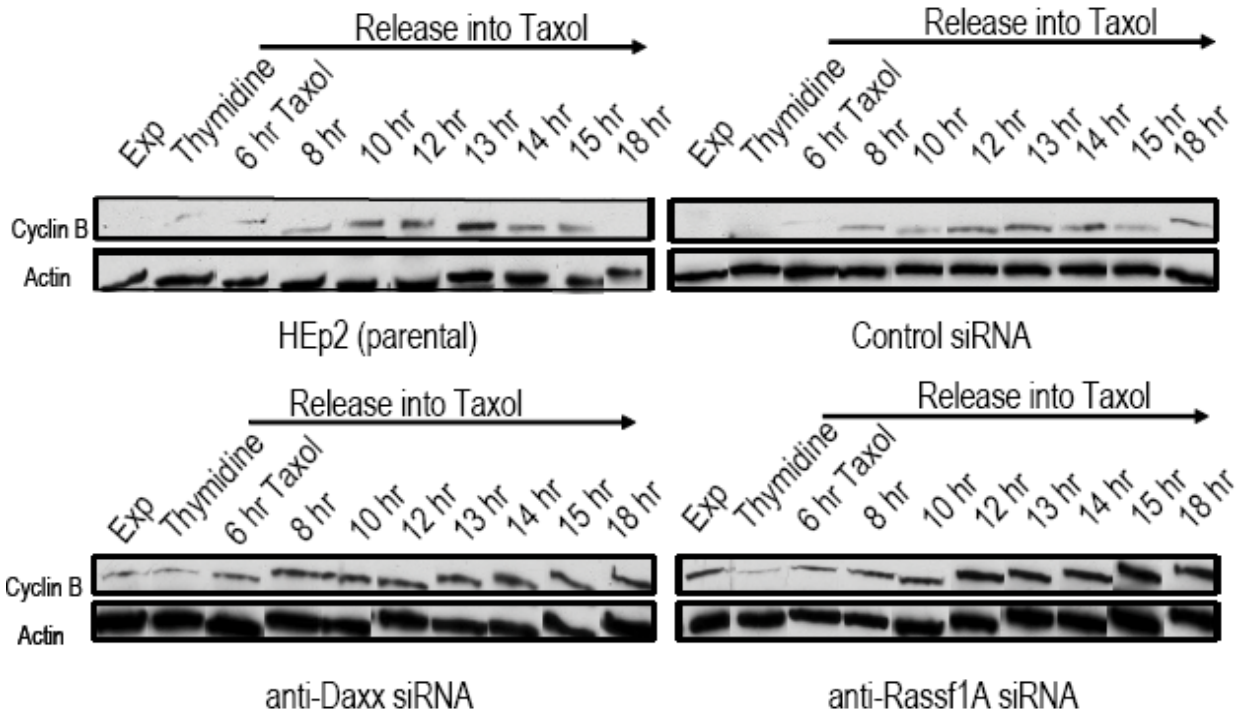


Figure 5-6.Cyclin B Levels are Stabilized in Daxx- and Rassf1A-Depleted Cells Treated with Taxol. Wild type (parental) HEp2, control and Daxx- and Rassf1A-depleted cell lines were synchronized with a double thymidine block, released and then exposed to taxol for 6-18hrs. Cells were harvested at the indicated time points and probed for Cyclin B1 levels using anti-Cyclin B1 antibody (Santa Cruz). Protein levels were normalized within each siRNA cell line. Note increased relative stability of cyclin B1 (normalized to actin) in both Daxx and Rassf1A-depleted cell lines.

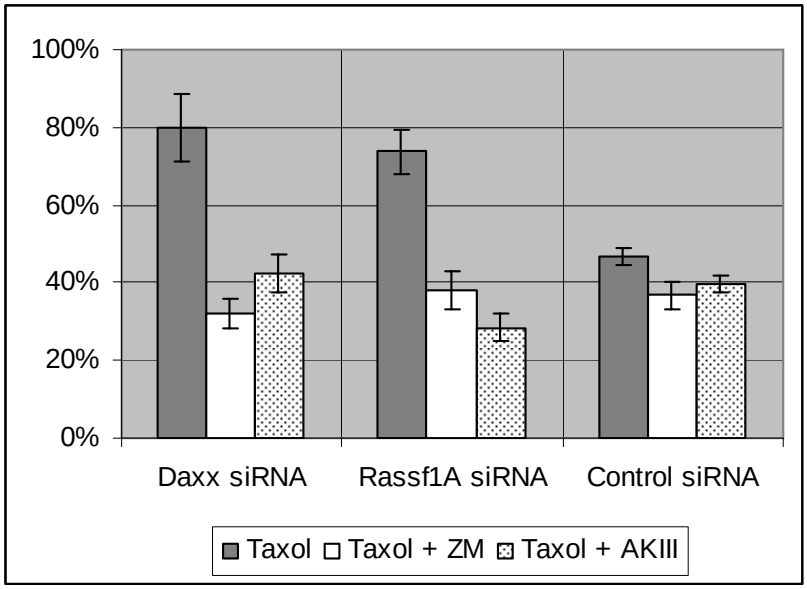


Figure 5-7. Inactivation of the Mitotic Spindle Checkpoint Using Aurora Kinase Inhibitors Abolishes Taxol Resistance in Daxx-and Rassf1A-Depleted Cells. Percentage of colonies that were formed from control, Daxx- and Rassf1A-depleted HEp2 cells which were synchronized using a double thymidine block, released and exposed to taxol alone, or taxol in combination with ZM447439 (ZM) or Aurora kinase inhibitor III (AKIII) for six hours is shown. Note taxol resistance is abolished in Daxx- and Rassf1A-depleted cells treated in combination with taxol and Aurora kinase inhibitors.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Taxane chemotherapy is considered among the most active treatment options for many breast cancer patients, either alone or as adjuvant in combination with anthracyclins (O'Shaughnessy, 2005). Nevertheless, a large number of patients are resistant to taxanes or become resistant to this therapy during treatment. The response rate of docetaxel is ~50% even after the first-line chemotherapy administration and it decreases to 20-30% in the second- or third-line administration (Bonneterre et al., 1999; Crown et al., 2004). A number of studies have been carried out to determine a genomic profile that could be predictive to taxane treatment (Chang et al., 2003; Chang et al., 2005b; Iwao-Koizumi et al., 2005; Mauriac et al., 2005; Miyoshi et al., 2004). However, an alternative approach to understand selective resistance to taxane treatment is to study mechanisms by which cells can respond to these drugs. Several molecular targets were reported, starting with mutations in α- and β-tubulin that affect drug binding, increased expression of tubulin genes, and changes in the synthesis or activity of tubulin interacting proteins (Hari et al., 2003a; Hari et al., 2003b; Wang and Cabral, 2005). Recently, a new class of potential targets are being studied after it was suggested that inactivation of mitotic proteins can contribute to the selective response of taxane treatment *in vivo* (Wassmann and Benezra, 2001). Thus, development of new genomic prognosis factors and in-depth understanding of drug activity on both a cellular and organismal level are needed for optimization of adjuvant therapy and proper patient stratification.

To this end, Daxx was identified as a novel regulator of paclitaxel response in cell culture conditions. Primary mouse and human fibroblasts with experimentally regulated levels of Daxx show both strong and divergent responses to taxol (Figure 4-1). In the absence of Daxx, cells remain in a prolonged mitotic block, while wild type cells undergo a transient arrest in mitosis

that is soon followed by micronucleation and cell death. Importantly, these observations were also recapitulated in MDA MB 468 and T47D breast cancer cell lines with contrasting levels of Daxx expression (Figure 4-3). These divergent responses in taxol response are usually seen in cells deficient in mitotic checkpoint proteins or other regulators of cell division and until these current studies, Daxx has not been implicated as a regulatory protein of cell cycle progression. Table 6-1 summarizes a growing list of mitotic proteins and the cellular response to taxol when these proteins are absent or deregulated in cells. To date, loss of function of the majority of mitotic proteins, including Mad1, Mad2, Bub1 and BubR1, has shown enhanced response to paclitaxel in cell culture conditions. Identification of factors which may increase drug resistance are largely uncharacterized. Among the conclusions of this study is that early mitosis progression is altered in cells lacking a functional Daxx protein. These observations came from tissue sectioning of $Daxx^{-/-}$ mouse embryos showing an increased number of cells in early mitotic stages (Figure 3-1) and were manifested from time-lapse microscopy studies analyzing mitotic progression in Daxx-depleted cell lines (Figure 3-3, Table 3-1) and Cyclin B protein stability studies showing an altered rate of cyclin degradation in anti-Daxx siRNA cells (Figure 3-4). Theoretically, alterations of this kind in cell cycle progression could, in part, explain the genomic instability that is observed in Daxx-deficient mouse cells (Figure 3-1) as improper mitosis frequently leads to unfaithful chromosome segregation (Chi and Jeang, 2007).

Daxx localization is already understood to be a very dynamic process during the cell cycle. During G1 and G2, Daxx localizes to PML bodies, while during S phase, it relocates to condensed heterochromatin where it interacts with ATRX, a chromatin remodeling protein. To accompany this dynamic protein trafficking, these studies have also revealed that Daxx, a known transcriptional regulator, can associate with the mitotic spindle apparatus during mitosis (Figure

3-7). Accruing evidence suggests that the tight orchestration of events during mitosis combines seemingly unrelated factors at critical junctures during cellular division (Tsai et al., 2006). A number of essential interactions occur during pro-metaphase when the nuclear envelope disintegrates and no longer compartmentalizes the nucleus and cytoplasm. During this time, Daxx is released from the nucleus where it is associated with ND10/PML bodies and accumulates at the mitotic spindle apparatus. It is at this spatiotemporal point that Daxx interacts with Rassf1 (Figure 5-4, Figure 6-1 summary). Future studies will reveal whether Daxx association with the spindle apparatus is dependent on presence of Rassf1.

To accompany observations of Daxx-dependent paclitaxel response, Rassf1 was identified as a novel Daxx-interacting protein that was also confirmed to be important for cellular taxol response (Figure 5-5). This interaction was mapped to the C spliceform of Rassf1 (Rassf1C) and interaction between Rassf1A and Rassf1C by dimerization was also confirmed (Figure 5-2). These novel interactions are thought to form a complex during mitosis and may perform critical regulatory processes including proper cellular response to mitotic stresses such as paclitaxel, nocodazole and other microtubule inhibiting compounds. These chemicals, moreover, can induce mitotic stress in several ways, depending on the stage of mitosis in which the stress is applied. Proper separation of the centrosomes during prophase, correct alignment of chromosomes during metaphase and faithful segregation of chromosomes during anaphase are all processes which can be altered or affected when external stress (i.e. paclitaxel) is applied. Together, Daxx and Rassf1 define a unique mitotic stress checkpoint during pro-metaphase (Figure 6-2, summary). Cells lacking Daxx or Rassf1A arrest in pro-metaphase during taxol treatment. From time-lapse studies, it is suggested that Daxx-depleted cells have chromosomes that remain unable to properly align at the metaphase plate under treatment conditions, thus

remaining in pro-metaphase by definition. In contrast, with normal primary cells and some tumor cell lines, Daxx and Rassf1A may be essential for proper mitotic exit in response to uncorrectable errors during pro-metaphase. This is suggested by the very robust response of wild type cells which only transiently arrest in pro-metaphase and then undergo micronucleation leading to cell death (Figure 4-3, Figure 4-6). One example of a mitotic stress checkpoint is already known with a protein named checkpoint with FHA and ring finger (CHFR). In the presence of CHFR, wild type cells exhibit a transient prophase arrest which temporarily prevents cells from entering metaphase under mitotic stress (Scolnick and Halazonetis, 2000). Cells that do not have a functional CHFR protein were shown to enter metaphase without delay and exhibited problems in proper centrosome separation. Thus, CHFR defines a prophase-specific mitotic stress checkpoint at a stage in mitosis earlier than Daxx and Rassf1.

Evidence accumulated in these studies suggests Daxx and Rassf1 are triggers for cellular taxol response. In the future, Daxx and Rassf1 may serve as ideal molecular markers for the proper selection of breast cancer patients (and other malignancies) for taxane chemotherapy. In order to achieve this goal, clinical studies will be required examining the status of Daxx and Rassf1 expression in tumors before and after taxane chemotherapy as well as in patients with an established history of taxane resistance. Altered Daxx or Rassf1 expression may be reminiscent of the differential protein expression that may exist in the original tumor cells from which each cell line was derived. It is already known that Daxx expression in some breast cancer cell lines is quite variable (Figure 4-1), but the extent of Daxx down-regulation or mutation in tumor cell lines has not been addressed. Rassf1A expression in tumor cell lines, conversely, has been extensively studied and shown to be altered in a majority of cases (Agathanggelou et al., 2005). Ultimately, these studies have established new roles for Daxx as a mitotic regulator that also

serves as a trigger for cellular taxol response in combination with Rassf1 which adds to our understanding of mechanisms linking cell division, genome instability and breast cancer progression.

Table 6-1. Alteration of Several Known Mitotic Proteins and Resultant Cellular Paclitaxel Response. Most mitotic proteins, when mutated or down-regulated in cells, display increased sensitivity to paclitaxel. In the absence of Daxx or Rassf1, cells display increased drug resistance.

Protein	Paclitaxel Response
Bub1	Increased Sensitivity (Lee et al., 2004; Sudo et al., 2004)
BubR1	Increased Sensitivity (Lee et al., 2004; Sudo et al., 2004)
CHFR	Increased Sensitivity (Satoh et al., 2003)
Mad1	Increased Sensitivity (Kienitz et al., 2005)
Mad2	Increased Sensitivity (Niikura et al., 2007)
Survivin	Increased Sensitivity (Carvalho et al., 2003)
Daxx	Increased Resistance (Lindsay et al., 2007)
Rassf1	Increased Resistance

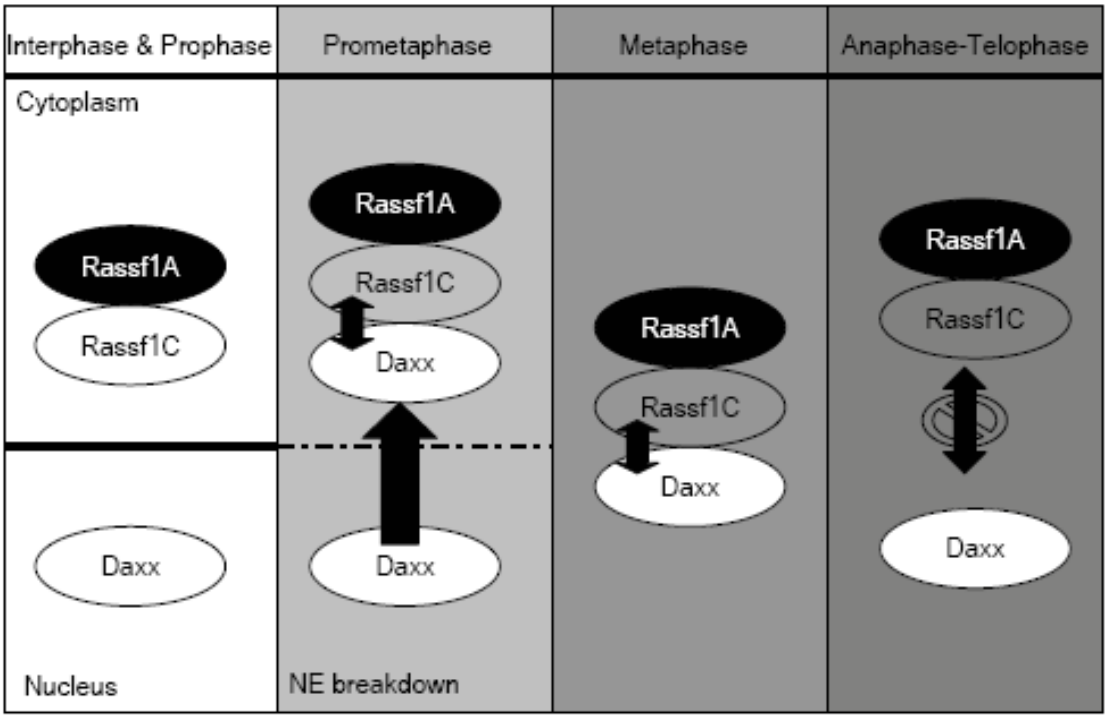


Figure 6-1. Dynamics of Daxx-Rassf1 Interaction Throughout the Cell Cycle. During interphase and beginning of mitosis (prophase), Daxx and Rassf1 are compartmentally separated in the cytoplasm and nucleus. After nuclear envelope (NE) breakdown, Daxx and Rassf1 can interact through Rassf1C. This association is maintained through metaphase, but by late stages of mitosis, is absent.

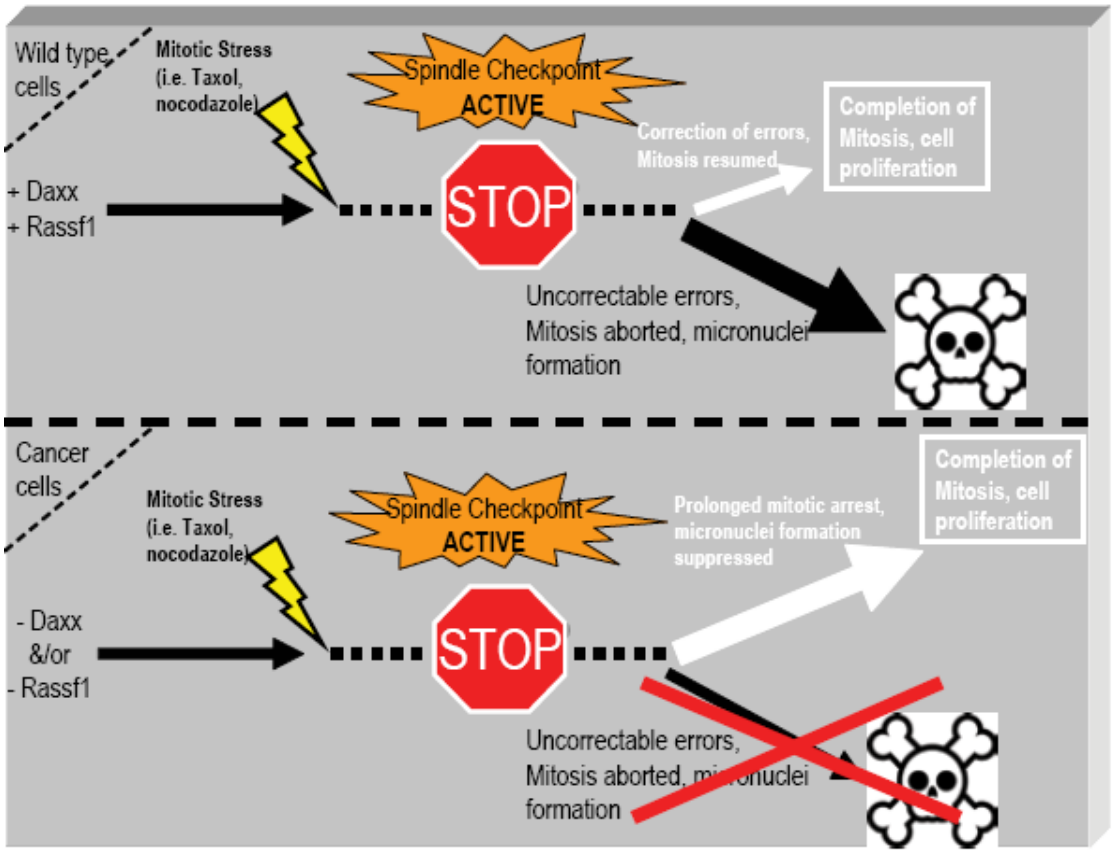


Figure 6-2. Model Depicting Daxx-Rassf1-Mediated Mitotic Stress Checkpoint During Pro-metaphase. In wild type cells upon mitotic stress (i.e. taxol or nocodazole) the spindle checkpoint will be activated. In rare cases, the cell may correct these errors, complete mitosis and resume cell proliferation. In most cases, however, these errors are uncorrected and the cell aborts mitosis, undergoes micronuclei formation and cell death. In subpopulations of cancer cells which encounter mitotic stress and which do not have a functional Daxx or Rassf1 protein, these cells remain in a prolonged mitotic block, unable to efficiently abort mitosis, and remain in pro-metaphase until drug removal or decay. This model may partially explain inherent and acquired taxol resistance in some breast tumors.

REFERENCES

Aapro, M.S. (2001). Neoadjuvant therapy in breast cancer: can we define its role? Oncologist *6 Suppl 3*, 36-39.

Agathanggelou, A., Cooper, W.N., and Latif, F. (2005). Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res *65*, 3497-3508.

Agnese, V., Bazan, V., Fiorentino, F.P., Fanale, D., Badalamenti, G., Colucci, G., Adamo, V., Santini, D., and Russo, A. (2007). The role of Aurora-A inhibitors in cancer therapy. Ann Oncol *18 Suppl 6*, vi47-52.

Amaar, Y.G., Minera, M.G., Hatran, L.K., Strong, D.D., Mohan, S., and Reeves, M.E. (2006). Ras association domain family 1C protein stimulates human lung cancer cell proliferation. Am J Physiol Lung Cell Mol Physiol *291*, L1185-1190.

Bonneterre, J., Spielman, M., Guastalla, J.P., Marty, M., Viens, P., Chollet, P., Roche, H., Fumoleau, P., Mauriac, L., Bourgeois, H., *et al.* (1999). Efficacy and safety of docetaxel (Taxotere) in heavily pretreated advanced breast cancer patients: the French compassionate use programme experience. Eur J Cancer *35*, 1431-1439.

Bothos, J., Summers, M.K., Venere, M., Scolnick, D.M., and Halazonetis, T.D. (2003). The Chfr mitotic checkpoint protein functions with Ubc13-Mms2 to form Lys63-linked polyubiquitin chains. Oncogene *22*, 7101-7107.

Brown, J.M., and Wouters, B.G. (1999). Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res *59*, 1391-1399.

Burbee, D.G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., *et al.* (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst *93*, 691-699.

Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K., Markowitz, S.D., Kinzler, K.W., and Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. Nature *392*, 300-303.

Carvalho, A., Carmena, M., Sambade, C., Earnshaw, W.C., and Wheatley, S.P. (2003). Survivin is required for stable checkpoint activation in taxol-treated HeLa cells. J Cell Sci *116*, 2987-2998.

Chabalier, C., Lamare, C., Racca, C., Privat, M., Valette, A., and Larminat, F. (2006). BRCA1 downregulation leads to premature inactivation of spindle checkpoint and confers paclitaxel resistance. Cell Cycle *5*, 1001-1007.

Chan, G.K., and Yen, T.J. (2003). The mitotic checkpoint: a signaling pathway that allows a single unattached kinetochore to inhibit mitotic exit. Prog Cell Cycle Res *5*, 431-439.

Chang, C.C., Lin, D.Y., Fang, H.I., Chen, R.H., and Shih, H.M. (2005a). Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. J Biol Chem *280*, 10164-10173.

Chang, J.C., Wooten, E.C., Tsimelzon, A., Hilsenbeck, S.G., Gutierrez, M.C., Elledge, R., Mohsin, S., Osborne, C.K., Chamness, G.C., Allred, D.C., and O'Connell, P. (2003). Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet *362*, 362-369.

Chang, J.C., Wooten, E.C., Tsimelzon, A., Hilsenbeck, S.G., Gutierrez, M.C., Tham, Y.L., Kalidas, M., Elledge, R., Mohsin, S., Osborne, C.K.*, et al.* (2005b). Patterns of resistance and incomplete response to docetaxel by gene expression profiling in breast cancer patients. J Clin Oncol *23*, 1169-1177.

Chaturvedi, P., Sudakin, V., Bobiak, M.L., Fisher, P.W., Mattern, M.R., Jablonski, S.A., Hurle, M.R., Zhu, Y., Yen, T.J., and Zhou, B.B. (2002). Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. Cancer Res *62*, 1797-1801.

Chen, E.S., Saitoh, S., Yanagida, M., and Takahashi, K. (2003). A cell cycle-regulated GATA factor promotes centromeric localization of CENP-A in fission yeast. Mol Cell *11*, 175-187.

Chi, Y.H., and Jeang, K.T. (2007). Aneuploidy and cancer. J Cell Biochem *102*, 531-538.

Cleveland, D.W., Mao, Y., and Sullivan, K.F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell *112*, 407-421.

Concin, N., Zeillinger, C., Tong, D., Stimpfl, M., Konig, M., Printz, D., Stonek, F., Schneeberger, C., Hefler, L., Kainz, C.*, et al.* (2003). Comparison of p53 mutational status with mRNA and protein expression in a panel of 24 human breast carcinoma cell lines. Breast Cancer Res Treat *79*, 37-46.

Crown, J., O'Leary, M., and Ooi, W.S. (2004). Docetaxel and paclitaxel in the treatment of breast cancer: a review of clinical experience. Oncologist *9 Suppl 2*, 24-32.

Croxton, R., Puto, L.A., de Belle, I., Thomas, M., Torii, S., Hanaii, F., Cuddy, M., and Reed, J.C. (2006). Daxx represses expression of a subset of antiapoptotic genes regulated by nuclear factor-kappaB. Cancer Res *66*, 9026-9035.

Dallol, A., Agathanggelou, A., Fenton, S.L., Ahmed-Choudhury, J., Hesson, L., Vos, M.D., Clark, G.J., Downward, J., Maher, E.R., and Latif, F. (2004). RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Res *64*, 4112-4116.

Dammann, R., Li, C., Yoon, J.H., Chin, P.L., Bates, S., and Pfeifer, G.P. (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet *25*, 315-319.

Dammann, R., Yang, G., and Pfeifer, G.P. (2001). Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res *61*, 3105-3109.

Dellaire, G., Eskiw, C.H., Dehghani, H., Ching, R.W., and Bazett-Jones, D.P. (2006). Mitotic accumulations of PML protein contribute to the re-establishment of PML nuclear bodies in G1. J Cell Sci *119*, 1034-1042.

Ditchfield, C., Johnson, V.L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S.S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol *161*, 267-280.

Donninger, H., Vos, M.D., and Clark, G.J. (2007). The RASSF1A tumor suppressor. J Cell Sci *120*, 3163-3172.

Ecsedy, J.A., Michaelson, J.S., and Leder, P. (2003). Homeodomain-interacting protein kinase 1 modulates Daxx localization, phosphorylation, and transcriptional activity. Mol Cell Biol *23*, 950-960.

Emelyanov, A.V., Kovac, C.R., Sepulveda, M.A., and Birshtein, B.K. (2002). The interaction of Pax5 (BSAP) with Daxx can result in transcriptional activation in B cells. J Biol Chem *277*, 11156-11164.

Fenton, S.L., Dallol, A., Agathanggelou, A., Hesson, L., Ahmed-Choudhury, J., Baksh, S., Sardet, C., Dammann, R., Minna, J.D., Downward, J., *et al.* (2004). Identification of the E1A-regulated transcription factor p120 E4F as an interacting partner of the RASSF1A candidate tumor suppressor gene. Cancer Res *64*, 102-107.

Fu, J., Bian, M., Jiang, Q., and Zhang, C. (2007). Roles of Aurora kinases in mitosis and tumorigenesis. Mol Cancer Res *5*, 1-10.

Giannakakou, P., Robey, R., Fojo, T., and Blagosklonny, M.V. (2001). Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. Oncogene *20*, 3806-3813.

Gostissa, M., Morelli, M., Mantovani, F., Guida, E., Piazza, S., Collavin, L., Brancolini, C., Schneider, C., and Del Sal, G. (2004). The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. J Biol Chem *279*, 48013-48023.

Hannemann, J., Oosterkamp, H.M., Bosch, C.A., Velds, A., Wessels, L.F., Loo, C., Rutgers, E.J., Rodenhuis, S., and van de Vijver, M.J. (2005). Changes in gene expression associated with response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol *23*, 3331-3342.

Hari, M., Wang, Y., Veeraraghavan, S., and Cabral, F. (2003a). Mutations in alpha- and beta-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine. Mol Cancer Ther *2*, 597-605.

Hari, M., Yang, H., Zeng, C., Canizales, M., and Cabral, F. (2003b). Expression of class III beta-tubulin reduces microtubule assembly and confers resistance to paclitaxel. Cell Motil Cytoskeleton *56*, 45-56.

Haruki, N., Saito, H., Harano, T., Nomoto, S., Takahashi, T., Osada, H., and Fujii, Y. (2001). Molecular analysis of the mitotic checkpoint genes BUB1, BUBR1 and BUB3 in human lung cancers. Cancer Lett *162*, 201-205.

Henderson, I.C., Berry, D.A., Demetri, G.D., Cirrincione, C.T., Goldstein, L.J., Martino, S., Ingle, J.N., Cooper, M.R., Hayes, D.F., Tkaczuk, K.H., *et al.* (2003). Improved outcomes from adding sequential Paclitaxel but not from escalating Doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. J Clin Oncol *21*, 976-983.

Hernandez-Vargas, H., Palacios, J., and Moreno-Bueno, G. (2006). Molecular profiling of docetaxel cytotoxicity in breast cancer cells: uncoupling of aberrant mitosis and apoptosis. Oncogene.

Hollenbach, A.D., McPherson, C.J., Mientjes, E.J., Iyengar, R., and Grosveld, G. (2002). Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. J Cell Sci *115*, 3319-3330.

Ikui, A.E., Yang, C.P., Matsumoto, T., and Horwitz, S.B. (2005). Low concentrations of taxol cause mitotic delay followed by premature dissociation of p55CDC from Mad2 and BubR1 and abrogation of the spindle checkpoint, leading to aneuploidy. Cell Cycle *4*, 1385-1388.

Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F., 3rd, and Maul, G.G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. J Cell Biol *147*, 221-234.

Ishov, A.M., Vladimirova, O.V., and Maul, G.G. (2004). Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX. J Cell Sci *117*, 3807-3820.

Iwao-Koizumi, K., Matoba, R., Ueno, N., Kim, S.J., Ando, A., Miyoshi, Y., Maeda, E., Noguchi, S., and Kato, K. (2005). Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin Oncol *23*, 422-431.

Jiang, N., Wang, X., Yang, Y., and Dai, W. (2006). Advances in mitotic inhibitors for cancer treatment. Mini Rev Med Chem *6*, 885-895.

Jung, Y.S., Kim, H.Y., Kim, J., Lee, M.G., Pouyssegur, J., and Kim, E. (2008). Physical interactions and functional coupling between Daxx and sodium hydrogen exchanger 1 in ischemic cell death. J Biol Chem *283*, 1018-1025.

Jung, Y.S., Kim, H.Y., Lee, Y.J., and Kim, E. (2007). Subcellular localization of Daxx determines its opposing functions in ischemic cell death. FEBS Lett *581*, 843-852.

Junn, E., Taniguchi, H., Jeong, B.S., Zhao, X., Ichijo, H., and Mouradian, M.M. (2005). Interaction of DJ-1 with Daxx inhibits apoptosis signal-regulating kinase 1 activity and cell death. Proc Natl Acad Sci U S A *102*, 9691-9696.

Kang, D., Chen, J., Wong, J., and Fang, G. (2002). The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. J Cell Biol *156*, 249-259.

Karunakaran, S., Diwakar, L., Saeed, U., Agarwal, V., Ramakrishnan, S., Iyengar, S., and Ravindranath, V. (2007). Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid. FASEB J *21*, 2226-2236.

Keen, N., and Taylor, S. (2004). Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer *4*, 927-936.

Khelifi, A.F., D'Alcontres, M.S., and Salomoni, P. (2005). Daxx is required for stress-induced cell death and JNK activation. Cell Death Differ *12*, 724-733.

Kienitz, A., Vogel, C., Morales, I., Muller, R., and Bastians, H. (2005). Partial downregulation of MAD1 causes spindle checkpoint inactivation and aneuploidy, but does not confer resistance towards taxol. Oncogene *24*, 4301-4310.

Kiriakidou, M., Driscoll, D.A., Lopez-Guisa, J.M., and Strauss, J.F., 3rd (1997). Cloning and expression of primate Daxx cDNAs and mapping of the human gene to chromosome 6p21.3 in the MHC region. DNA Cell Biol *16*, 1289-1298.

Kitagawa, D., Kajiho, H., Negishi, T., Ura, S., Watanabe, T., Wada, T., Ichijo, H., Katada, T., and Nishina, H. (2006). Release of RASSF1C from the nucleus by Daxx degradation links DNA damage and SAPK/JNK activation. EMBO J *25*, 3286-3297.

Ko, Y.G., Kang, Y.S., Park, H., Seol, W., Kim, J., Kim, T., Park, H.S., Choi, E.J., and Kim, S. (2001). Apoptosis signal-regulating kinase 1 controls the proapoptotic function of death-associated protein (Daxx) in the cytoplasm. J Biol Chem *276*, 39103-39106.

Kroemer, G., and Martin, S.J. (2005). Caspase-independent cell death. Nat Med *11*, 725-730.

Lalioti, V.S., Vergarajauregui, S., Pulido, D., and Sandoval, I.V. (2002). The insulin-sensitive glucose transporter, GLUT4, interacts physically with Daxx. Two proteins with capacity to bind Ubc9 and conjugated to SUMO1. J Biol Chem *277*, 19783-19791.

Lee, E.A., Keutmann, M.K., Dowling, M.L., Harris, E., Chan, G., and Kao, G.D. (2004). Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells. Mol Cancer Ther *3*, 661-669.

Lehembre, F., Muller, S., Pandolfi, P.P., and Dejean, A. (2001). Regulation of Pax3 transcriptional activity by SUMO-1-modified PML. Oncogene *20*, 1-9.

Lens, S.M., Wolthuis, R.M., Klompmaker, R., Kauw, J., Agami, R., Brummelkamp, T., Kops, G., and Medema, R.H. (2003). Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. Embo J *22*, 2934-2947.

Li, H., Leo, C., Zhu, J., Wu, X., O'Neil, J., Park, E.J., and Chen, J.D. (2000). Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. Mol Cell Biol *20*, 1784-1796.

Li, W., Zhu, T., and Guan, K.L. (2004). Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK. J Biol Chem *279*, 37398-37406.

Lin, D.Y., Lai, M.Z., Ann, D.K., and Shih, H.M. (2003). Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential. J Biol Chem *278*, 15958-15965.

Lindsay, C.R., Scholz, A., Morozov, V.M., and Ishov, A.M. (2007). Daxx shortens mitotic arrest caused by paclitaxel. Cell Cycle *6*, 1200-1204.

Liu, L., Amy, V., Liu, G., and McKeehan, W.L. (2002). Novel complex integrating mitochondria and the microtubular cytoskeleton with chromosome remodeling and tumor suppressor RASSF1 deduced by in silico homology analysis, interaction cloning in yeast, and colocalization in cultured cells. In Vitro Cell Dev Biol Anim *38*, 582-594.

Liu, L., Baier, K., Dammann, R., and Pfeifer, G.P. (2007). The tumor suppressor RASSF1A does not interact with Cdc20, an activator of the anaphase-promoting complex. Cell Cycle *6*, 1663-1665.

Liu, L., Tommasi, S., Lee, D.H., Dammann, R., and Pfeifer, G.P. (2003). Control of microtubule stability by the RASSF1A tumor suppressor. Oncogene *22*, 8125-8136.

Malumbres, M. (2006). Therapeutic opportunities to control tumor cell cycles. Clin Transl Oncol *8*, 399-408.

Mansilla, S., Bataller, M., and Portugal, J. (2006a). Mitotic catastrophe as a consequence of chemotherapy. Anticancer Agents Med Chem *6*, 589-602.

Mansilla, S., Priebe, W., and Portugal, J. (2006b). Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. Cell Cycle *5*, 53-60.

Mauriac, L., Debled, M., and MacGrogan, G. (2005). When will more useful predictive factors be ready for use? Breast *14*, 617-623.

Meraldi, P., Draviam, V.M., and Sorger, P.K. (2004). Timing and checkpoints in the regulation of mitotic progression. Dev Cell *7*, 45-60.

Michaelson, J.S. (2000). The Daxx enigma. Apoptosis *5*, 217-220.

Michaelson, J.S., Bader, D., Kuo, F., Kozak, C., and Leder, P. (1999). Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev *13*, 1918-1923.

Michaelson, J.S., and Leder, P. (2003). RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. J Cell Sci *116*, 345-352.

Miyoshi, Y., Kim, S.J., Akazawa, K., Kamigaki, S., Ueda, S., Yanagisawa, T., Inoue, T., Taguchi, T., Tamaki, Y., and Noguchi, S. (2004). Down-regulation of intratumoral aromatase messenger RNA levels by docetaxel in human breast cancers. Clin Cancer Res *10*, 8163-8169.

Morrow, C.J., Tighe, A., Johnson, V.L., Scott, M.I., Ditchfield, C., and Taylor, S.S. (2005). Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. J Cell Sci *118*, 3639-3652.

Morse, D.L., Gray, H., Payne, C.M., and Gillies, R.J. (2005). Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. Mol Cancer Ther *4*, 1495-1504.

Muromoto, R., Sugiyama, K., Takachi, A., Imoto, S., Sato, N., Yamamoto, T., Oritani, K., Shimoda, K., and Matsuda, T. (2004). Physical and functional interactions between Daxx and DNA methyltransferase 1-associated protein, DMAP1. J Immunol *172*, 2985-2993.

Musacchio, A., and Salmon, E.D. (2007). The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol *8*, 379-393.

Nefkens, I., Negorev, D.G., Ishov, A.M., Michaelson, J.S., Yeh, E.T., Tanguay, R.M., Muller, W.E., and Maul, G.G. (2003). Heat shock and Cd2+ exposure regulate PML and Daxx release from ND10 by independent mechanisms that modify the induction of heat-shock proteins 70 and 25 differently. J Cell Sci *116*, 513-524.

O'Shaughnessy, J. (2005). Extending survival with chemotherapy in metastatic breast cancer. Oncologist *10 Suppl 3*, 20-29.

Perlman, R., Schiemann, W.P., Brooks, M.W., Lodish, H.F., and Weinberg, R.A. (2001). TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. Nat Cell Biol *3*, 708-714.

Pines, J. (2006). Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol *16*, 55-63.

Pluta, A.F., Earnshaw, W.C., and Goldberg, I.G. (1998). Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. J Cell Sci *111 (Pt 14)*, 2029-2041.

Ravdin, P., Erban, J., and Overmoyer, B. (2003). Phase III comparison of docetaxel and paclitaxel in patients with metastatic breast cancer. Eur J Cancer *suppl1:32*.

Ricci, M.S., and Zong, W.X. (2006). Chemotherapeutic approaches for targeting cell death pathways. Oncologist *11*, 342-357.

Rong, R., Jin, W., Zhang, J., Sheikh, M.S., and Huang, Y. (2004). Tumor suppressor RASSF1A is a microtubule-binding protein that stabilizes microtubules and induces G2/M arrest. Oncogene *23*, 8216-8230.

Roninson, I.B., Broude, E.V., and Chang, B.D. (2001). If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat *4*, 303-313.

Rowinsky, E.K., Eisenhauer, E.A., Chaudhry, V., Arbuck, S.G., and Donehower, R.C. (1993). Clinical toxicities encountered with paclitaxel (Taxol). Semin Oncol *20*, 1-15.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. Nat Med *11*, 1306-1313.

Schiff, P.B., Fant, J., and Horwitz, S.B. (1979). Promotion of microtubule assembly in vitro by taxol. Nature *277*, 665-667.

Scolnick, D.M., and Halazonetis, T.D. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. Nature *406*, 430-435.

Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R., and White, M.A. (2002). The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol Cell Biol *22*, 4309-4318.

Song, J.J., and Lee, Y.J. (2003). Role of the ASK1-SEK1-JNK1-HIPK1 signal in Daxx trafficking and ASK1 oligomerization. J Biol Chem *278*, 47245-47252.

Song, J.J., and Lee, Y.J. (2004). Tryptophan 621 and serine 667 residues of Daxx regulate its nuclear export during glucose deprivation. J Biol Chem *279*, 30573-30578.

Song, M.S., Song, S.J., Ayad, N.G., Chang, J.S., Lee, J.H., Hong, H.K., Lee, H., Choi, N., Kim, J., Kim, H., *et al.* (2004). The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. Nat Cell Biol *6*, 129-137.

Song, M.S., Song, S.J., Kim, S.J., Nakayama, K., Nakayama, K.I., and Lim, D.S. (2007). Skp2 regulates the antiproliferative function of the tumor suppressor RASSF1A via ubiquitin-mediated degradation at the G(1)-S transition. Oncogene.

Sudo, T., Nitta, M., Saya, H., and Ueno, N.T. (2004). Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. Cancer Res *64*, 2502-2508.

Tang, J., Qu, L.K., Zhang, J., Wang, W., Michaelson, J.S., Degenhardt, Y.Y., El-Deiry, W.S., and Yang, X. (2006). Critical role for Daxx in regulating Mdm2. Nat Cell Biol *8*, 855-862.

Torii, S., Egan, D.A., Evans, R.A., and Reed, J.C. (1999). Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). EMBO J *18*, 6037-6049.

Tsai, M.Y., Wang, S., Heidinger, J.M., Shumaker, D.K., Adam, S.A., Goldman, R.D., and Zheng, Y. (2006). A mitotic lamin B matrix induced by RanGTP required for spindle assembly. Science *311*, 1887-1893.

Vos, M.D., Martinez, A., Elam, C., Dallol, A., Taylor, B.J., Latif, F., and Clark, G.J. (2004). A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability. Cancer Res *64*, 4244-4250.

Wang, L.G., Liu, X.M., Kreis, W., and Budman, D.R. (1999). The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemother Pharmacol *44*, 355-361.

Wang, Y., and Cabral, F. (2005). Paclitaxel resistance in cells with reduced beta-tubulin. Biochim Biophys Acta *1744*, 245-255.

Wassmann, K., and Benezra, R. (2001). Mitotic checkpoints: from yeast to cancer. Curr Opin Genet Dev *11*, 83-90.

Wood, K.W., Cornwell, W.D., and Jackson, J.R. (2001). Past and future of the mitotic spindle as an oncology target. Curr Opin Pharmacol *1*, 370-377.

Xia, G., Luo, X., Habu, T., Rizo, J., Matsumoto, T., and Yu, H. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. Embo J *23*, 3133-3143.

Xue, Y., Gibbons, R., Yan, Z., Yang, D., McDowell, T.L., Sechi, S., Qin, J., Zhou, S., Higgs, D., and Wang, W. (2003). The ATRX syndrome protein forms a chromatin-remodeling complex with Daxx and localizes in promyelocytic leukemia nuclear bodies. Proc Natl Acad Sci U S A *100*, 10635-10640.

Yang, X., Khosravi-Far, R., Chang, H.Y., and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Yeung, T.K., Germond, C., Chen, X., and Wang, Z. (1999). The mode of action of taxol: apoptosis at low concentration and necrosis at high concentration. Biochem Biophys Res Commun *263*, 398-404.

Yu, X., Minter-Dykhouse, K., Malureanu, L., Zhao, W.M., Zhang, D., Merkle, C.J., Ward, I.M., Saya, H., Fang, G., van Deursen, J., and Chen, J. (2005). Chfr is required for tumor suppression and Aurora A regulation. Nat Genet *37*, 401-406.

Zhao, L.Y., Liu, J., Sidhu, G.S., Niu, Y., Liu, Y., Wang, R., and Liao, D. (2004). Negative regulation of p53 functions by Daxx and the involvement of MDM2. J Biol Chem *279*, 50566-50579.

Zhong, S., Salomoni, P., Ronchetti, S., Guo, A., Ruggero, D., and Pandolfi, P.P. (2000). Promyelocytic leukemia protein (PML) and Daxx participate in a novel nuclear pathway for apoptosis. J Exp Med *191*, 631-640.

BIOGRAPHICAL SKETCH

Cory Lindsay was born in Laurel, Nebraska, a small town located in northeast Nebraska cornering Iowa and South Dakota. He is the son of Archie and Virginia Lindsay, of Laurel, and has three siblings Lori, Scott and Michelle. Cory attended Laurel-Concord High School and graduated in 1998. During his high school years, Cory had several outstanding teachers who motivated and inspired him to pursue academics. His first experience with science research came during this time when he studied meiofauna biology and won several local, regional and national awards for his efforts. He also developed an interest in collecting and breeding snakes and other reptiles and amphibians, a hobby which he still pursues today.

After high school, Cory pursued a Bachelor of Science degree in Biological Sciences from Wayne State College, in Wayne, NE and graduated in 2002. While an undergraduate, Cory was fortunate to have many opportunities to perform independent science research. He conducted ecological research in the British West Indies studying dwarf-geckos and snakes as part of a collaborative effort with Avila College, Kansas City, MO; identified novel genes from the *Schistosoma mansoni* genome at the Whitney Laboratory, St. Augustine, FL; performed molecular biology experiments as an intern at the Bermuda Biological Station for Research, St. Georges, Bermuda; and deciphered genetic disease abnormalities at the world famous Cold Spring Harbor Laboratory, Long Island, NY.

Cory joined the University of Florida's IDP graduate school program in 2003 and finished his Ph.D work in the laboratory of Dr. Alexander Ishov studying Daxx function in cellular taxol response and cell cycle progression. He has presented this work at several national and international conferences and published his work summarizing these results. Cory will continue post-doctoral work in cancer research and diagnostics.